THE ULTRASTRUCTURE OF HUMAN CHROMOSOMES

by

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INTRODUCTION

Statement of the problem

In recent years much has been learned about human chromosomes, but the structural organization of nucleic acids and associated proteins within these chromosomes remains poorly understood. One direct means of securing information to resolve this problem is by electron microscopy, and the chromosomes of a number of eukaryote species have been studied (1-18). However, the complexity of these chromosomes makes interpretation of results difficult, and there has been no general agreement on structural models of eukaryote chromosomes. In addition, only a few investigations have dealt with unsectioned human chromosomes (4,10,17, 18). Therefore an electron microscopic examination of the chromosomes of cultured normal human lymphocytes at various stages of mitosis was undertaken, and the goal was to characterize chromosome fibers and to define organizational patterns of these fibers within chromosomes.

Review of the literature

Chromosomes carry the genetic material

Chromosomes and chromosome components have concerned biochemists, cytologists, and geneticists for nearly one hundred years. Initial contributions to the knowledge of chromosomes were made by Miescher in 1869 who discovered that pus cells contain nucleic acids (19), by Flemming in 1882 who described the mitotic cycle (20), and by Waldeyer in 1888 who gave the term "chromosome" to the deeply staining material

within the nucleus which displayed characteristic behavior when the cell divided (21). It was not until 1902, two years after the rediscovery of Mendel's laws of segregation and independent assortment, that Sutton suggested that the physical basis of heredity might be found in the behavior of chromosomes during meiosis (22). In the 1920's and 1930's studies which employed cytochemical and ultraviolet microscopy techniques contributed fundamental information about the chemical components of chromosomes. Feulgen in 1924 described a cytochemical reaction which demonstrated the presence of deoxyribonucleic acid (DNA) in cell nuclei and chromosomes but not in cytoplasm (23). Caspersson first recognized the unique absorption spectrum of nucleic acids in ultraviolet light, and in 1936 he demonstrated that nucleic acid dominated the ultraviolet absorption in a chromosome to such an extent that the absorption spectrum of a metaphase chromosome was almost like that of a pure nucleic acid. By this method he demonstrated the presence of nucleic acid and protein in giant salivary gland chromosomes of Drosophila melanogaster (24).

The concept that DNA is the genetic material arose from two areas of investigation: studies which correlated the properties of DNA and the genetic material and studies of transformation. In 1939 Hollaender (25) and Emmons and Hollaender (26) observed that the ultraviolet action spectrum for mutation was similar to the ultraviolet absorption spectrum for nucleic acids which implicated nucleic acid as the genetic material. Boiven, Vendrely, and Vendrely in 1948 inferred from the bacterial transforming principle that DNA must be very close to the actual genetic material and hypothesized that its distribution should run parallel to

that of the chromosome and gene cycle. Experimentally they found that the DNA content per nucleus in various beef organs was nearly the same and that approximately one-half this amount of DNA was present in haploid nuclei of bull sperm. DNA was found almost entirely within the nucleus and the amount of DNA quantitatively correlated with the number of chromosomes (27). Litman and Pardee in 1956 reported an increased mutation rate following incorporation of 5-bromouracil, a thymine analogue, into DNA (28). Transformation studies began in 1928 when Griffith observed that living smooth forms of pneumococcus could be recovered from mice which had been injected with a mixture of living rough forms and killed smooth forms (29). In 1944 Avery, Macleod, and McCarty isolated the factor causing genetic transformation of characters in bacteria in nearly pure form and identified it as DNA (30). Hotchkiss and Marmur in 1954 demonstrated linked transfer of two genetic markers when wild-type pneumococci were placed in a medium which contained pure DNA from pneumococci that carried the two markers (31). A detailed account of the chromosome and its history has been prepared by Grell (32).

Composition of chromosomes

In addition to DNA and proteins, chromosomes contain ribonucleic acid (RNA), lipids, cations, and water. The quantity of all of these substances except water has been determined in isolated nuclei, interphase chromosomes, and metaphase chromosomes; and these data which are expressed on a dry weight basis are summarized in Table 1. The total protein:DNA ratio in nuclei is about 1.9, whereas in metaphase chromosomes it is 5.0. The histone:DNA ratio in nuclei is about 1.5 and in metaphase

chromosomes is 3.0. To provide a basis for later discussions of chromosome structure each of these components will be discussed in terms of its primary, secondary, and tertiary structure (when applicable) and of its interaction with other chromosome components. Water which probably accounts for about 75% of the weight of these structures in vivo will be included in some of these discussions, but in general there is a dearth of information concerning its role in chromosome structure.

DNA is the fundamental carrier of genetic information in eukaryote species and serves both repository (conservation and propagation of genetic material) and transcriber (cellular metabolism) functions (11). In 1953 Watson and Crick proposed a molecular model for DNA and subsequently proposed certain genetic mechanisms which were implied by the model (40-42). Since that time these hypotheses have been tested and proven correct. DNA is a polymer of deoxyribonucleotides, and each nucleotide consists of a deoxyribose sugar, a phosphate group, and a purine or pyrimidine base. The sugar and phosphate molecules form the backbone of the chain through 3',5' phosphodiester linkages, and the purine (adenine and guanine) and pyrimidine (thymine and cytosine) base sequences form the genetic code (41). Each such long DNA chain is joined to another chain by hydrogen bonding between specific complementary bases (adenine to thymine and guanine to cytosine), and the two strands, having opposite polarity, form right-handed helical coils around a common axis. When DNA replicates the two strands separate, and each strand acts as a template for the synthesis of a daughter strand which then contains complementary bases (41). The molecular dimensions and configurations of DNA were determined by x-ray diffraction, and three forms A, B, and C have been

observed (43). The Watson-Crick model is based on the paracrystalline B configuration which has been found in a wide range of species, which occurs when there is more than 30% water content in the molecule, which probably exists when DNA is in solution in water, and which is observed in vivo (43). In this form the phosphate and sugar molecules, which are on the outside of the molecule in all three forms, are 10 Å from the helix axis, and the overall diameter of the double helix is 20 Å. The distance between each nucleotide is 3.4 Å, and there are ten such intervals (34 Å) and ten nucleotides for every complete turn of the helix (40). Long fibers with 20 Å diameters have also been observed in electron micrographs of DNA (44-46). The average molecular weight of natural DNA as determined by light scattering is between 6×10^6 and 10×10^6 (47). This molecule would contain approximately 3×10^4 nucleotides (42) and would be about four micra in length when in the form of an uninterrupted Watson-Crick helix (46). The interaction of DNA with other chromosomal constituents and its arrangement within chromosomes will be discussed in subsequent paragraphs.

Proteins are intimately associated with DNA in the chromosomes of eukaryote species (9), and these proteins include histones, non-histone proteins, and protamines. In recent years much research activity has been directed toward these proteins, particularly the histones, in an effort to define their structural and functional roles in the genetic system (48-52). Only the histones and non-histone proteins will be considered in this review.

The histones which constitute the major portion of the chromosomal

proteins (Table 1) are the most basic proteins known apart from the protamines. Histones contain high proportions of arginine and lysine and have an isoelectric pH of 11 (48). They are extracted with dilute acid (0.1-0.3N hydrochloric or sulfuric acid) from whole tissue, isolated nuclei, or nucleoprotein; alternatively, nucleoprotein is dissociated in solutions of high salt concentration and then the histone is separated from the other components (mostly DNA) (48). Amino acid sequence determination is difficult because of the complexity of the histone fractions; however, a number of studies have confirmed a significant finding: basic amino acids do not occur at regular intervals in the peptide chains. This observation has not been made in just one fraction but in fraction Ia (molar ratio of arginine:lysine, 0.12; "very lysine rich") (52), fraction 2b (molar ratio of arginine:lysine, 0.5; "slightly lysine rich") (51), and the "arginine rich" fraction (molar ratio of arginine:lysine, greater than 1) (53). Since basic amino acids account for 25% of the residues in histones their average occurrence would be one in every four residues, but their actual occurrence could be XBXXXXBXXBXXXBXX where B is a basic amino acid and X is a non-basic amino acid (48). The basic amino acid composition of a fraction determines its affinity for polyanions, and if all the basic amino acid residues are involved in electrostatic linkages fractions Ia and Ib would have about 27 bonds, fraction IIb would have about 38 bonds, and fraction III would have at least 45 bonds (48,52). About 30% of the total histones are "very lysine rich" (Ia, Ib), 50% are "slightly lysine rich" (IIaa, IIa, IIb, IIc), and 20% are "arginine rich" (III, IV) (48). Configuration studies with optical rotatory

dispersion and infrared spectroscopy indicate that fraction Ib is completely unfolded in aqueous solution and that IIb and III have about 15 to 20% alpha helical content. All of these fractions exhibit increasing alpha helical content with increasing salt concentrations. The molecular weights of such fractions are 10,000 (Ia), 16,000 (IIb), and 26,000 (III) (52).

Stedman and Stedman in 1950 after observing some instances of cell and species specificity for histones, particuarly a marked difference in arginine content between histones of fowl erythrocytes and fowl thymus or spleen, proposed that histones might act as regulators of genetic activity by specific gene repression (54). Interest in this theory was rekindled in 1962 by Huang and Bonner who demonstrated in vitro that the addition of pea chromatin histone to DNA repressed its ability to act as a primer for RNA synthesis (55). This observation has been confirmed by others, and many subsequent investigations have dealt with various aspects of the histones. For further details the reader is referred to reviews of the subject (48-52).

The non-histone proteins (residual proteins) which occur in lesser quantities than histones in chromosomes and nuclei (Table 1) are not extracted from nucleoprotein by dilute acids (33,56-58). They are bound to DNA not by electrostatic bonds but by covalent bonds (57,58), and because they are relative polyanions they probably interact with histones (67). These proteins contain high proportions of glutamic acid (11-14%), aspartic acid (8-10%), leucine (9%), and glycine (9%) (51,58) and are classified as acidic nuclear proteins (51). In liver cell nuclei and Walker tumor cell nuclei glutamic and aspartic acid

comprise approximately 20-25% of the amino acid residues of the acidic proteins; and arginine, lysine, and histidine comprise about 16% of the residues (50). For rat liver nuclei the residual protein amino acid composition is qualitatively similar to histone from the same cells with the exception of cysteine which is not present in histone (58). The degree of heterogeneity of the residual protein is indicated by the number of NH_2-terminal amino acids. Busch and Steele have identified ten NH_2-terminal amino acids in this fraction from nuclei of Walker tumor and liver cells but noted that serine, alanine, and glycine together accounted for 55% of the total (50). Dounce and Hilgartner found lysine and threonine as NH_2-terminal amino acids in residual protein of rat liver nuclei and demonstrated two protein components by cellulose acetate electrophoresis (58). Thus the non-histone proteins are probably not a highly heterogeneous group. There have been no studies of amino acid sequence, molecular configuration, or molecular weight on these proteins. Dounce has estimated the molecular weight of the residual protein on the basis of cysteine content to be approximately 14,000 which corresponds to chain lengths of about 127 amino acids (58). Frenster has reported that non-histone residual proteins which contain 15% phosphoprotein are present in active chromatin (euchromatin) in a 2-fold excess as compared to inactive chromatin (heterochromatin) (67).

At this point it is appropriate to discuss nucleoprotein interactions. At least three forms of such interaction occur: one involves electrostatic bonds between polyanionic DNA and polycationic histone and is relatively labile; a second probably involves covalent bonds between DNA and residual (non-histone) proteins and is relatively stable; and a third

probably involves electrostatic bonds between histones and non-histone proteins and is relatively labile.

The interaction between DNA and histones is primarily electrostatic in nature. This is supported by the fact that at physiologic pH the DNA phosphate groups are anions and the histone arginine and lysine nitrogens are cations (48). Further evidence on this point comes from studies of DNA-histone dissociation in solutions of increasing salt concentration. At concentrations of 0.4M NaCl or less there is no dissociation; in 1.0M NaCl there is about 50% dissociation; and in 2.0M NaCl 75% of the histone is dissociated from DNA (62). Vendrely, et al. calculated that all groups of DNA can be saturated by the basic amino acids (arginine, lysine, histidine) which belong to the protein fraction extracted in 1.0M NaCl (34). Phillips calculated that a histone content by weight of 1.3 to 1.4 times the DNA content would be required to neutralize all of the DNA phosphate groups (48), and this requirement is met (Table 1). However there is very little excess basic amino acid and the non-regular occurrence of these residues in histone peptides (51-53) makes it improbable that all phosphate groups are neutralized by such residues.

The structure of deoxyribonucleoprotein (DNP) is not well understood. However it is known that DNA within the DNP maintains its twin-helical structure in aqueous solution (63), that a greater part of the protein is in the alpha-helical form and is distributed evenly along the DNA molecule perhaps along the larger groove of the helix (46,64) and that in oriented sheets of DNP films the DNA is probably in the B-form at relative humidities of 80% or more (65). Electron micrographs of DNP reveal a relatively uniform fiber with a diameter of 30±10 Å which supports the

view that histone is spread uniformly over the double stranded DNA structure (46,66). From light scattering determinations a molecular weight of about 18 X 10^6 was obtained for calf thymus DNP (62,46).

The relationship of non-histone proteins to other chromosomal components is poorly understood; however, at least two forms of interaction are probable. Dounce has proposed that in residual proteins polypeptides are joined to one another by disulfide linkages and that such proteins serve to link Watson-Crick DNA molecules end to end by forming covalent bonds with DNA (58). Frenster, after observing an excess of polyanionic residual protein in euchromatin (along with an excess of other polyanionic molecules), suggested that the substances interact with histones (polycations) electrostatically and thus cause histones to be less firmly bound to DNA (polyanion) (67).

The role of histones and other nuclear substances in gene regulation has received much attention recently. This literature will be discussed only briefly, and the reader is referred to references 35, 48-52 and 67 for a complete review. Because histones repress DNA dependent RNA synthesis they have been suggested to be gene regulators (55). It is probable that they are involved in gene regulation, but it is improbable that they are the primary regulating substance. A number of observations relate to this proposal. First, the histone-DNA interaction is primarily electrostatic, and although the primary structure of histone could give some specificity to such an interaction it is difficult to envision a highly specific relationship. Second, although the histones are heterogeneous proteins, and are probably somewhat cell specific (52), there are not enough codons in the genome to direct synthesis of specific

proteins for each gene. There must be a limited number of genes specifying synthesis of a limited number of histone molecules which then become common to a number of genes at the time of interaction (68). Third, Frenster and his associates have found nearly equal amounts of histone for the DNA in chromatin fractions which are active (euchromatin) and repressed (heterochromatin) with regard to RNA synthesis. However, they have found differences in polyanion content between these fractions. In euchromatin they find a two-fold excess of non-histone residual protein, a five-fold excess of RNA and of phospholipids, and a seven-fold excess of phosphoproteins. These and additional experiments led Frenster to propose that nuclear polyanions are de-repressors which antagonize the DNA-histone association and which effect a partial displacement of histone from DNA. This displacement allows intermittent separation of the strands of the DNA double helix which in turn allows RNA synthesis. The specificity of such a de-repressing system may reside in specific RNA (de-repressor)-DNA interaction and in the interaction of other molecules (e.g., hormones) with the polyanions of euchromatin (67).

Lipids are associated with nucleoprotein and comprise about 1 to 5% of the dry weight of isolated nuclei or chromatin fractions (59-61). Very little else was known until a recent study by Rose and Frenster (61) which elucidated the composition and metabolism of lipids within repressed and active chromatin of interphase lymphocytes. The total lipid content of isolated chromatin (euchromatin and heterochromatin) was 60.8 μg per mg of chromatin protein, or when converted to percentage, about 4% of the dry weight of isolated chromatin. The lipid composition was phospholipids (67.6 to 78.0%), neutral lipids (12.8 to 16.7%), and unidentified

polar lipid (7.1 to 15.8%). Analysis of the phospholipids indicated that lecithin was the predominant fraction (58.2 to 63.4%), phosphatidylethanolamine was next in quantity (27.8 to 31.5%), and sphingomyelin was present in small quantities. The presence of lipid in chromatin was confirmed by utilizing tritiated (^3H) glycerol for electron microscopic autoradiography. The lipid content among the subnuclear fractions was greater in euchromatin than in heterochromatin in every case. It was speculated by the authors that the combination of non-histone residual protein and phospholipid micelles as a lipoprotein polyanion may play a role in de-repressing repressed DNA by interacting with the histones (61).

The divalent cations calcium (Ca^{++}) and magnesium (Mg^{++}) are present in nuclei and chromosomes (Table 1), and their interaction with other chromosomal components is an important factor in chromosome structure. In aqueous solution Ca^{++} and Mg^{++} interact primarily with oxygen groups such as carboxyls and phosphates (69,70). At the macromolecular level Zubay and Doty observed aggregation and decreased viscosity of solutions of calf thymus deoxyribonucleoprotein (DNP) when small amounts of $Mg^{++}(10^{-5}M)$ were added (46). Frick observed reversible precipitation of sea urchin DNP solutions when $CaCl_2$ or $MgCl_2$ ($10^{-4.2}M$) were added and observed aggregation of DNP at lower salt concentrations. He calculated that the precipitated DNP contained one Ca^{++} per eleven nucleotides (71). With the electron microscope Maggio, et al. detected clumping and precipitation of nucleoplasm when the cell fixative solutions contained greater than 1.5mM $CaCl_2$ (72). Removal of divalent cations causes dissociation of DNP, and Solari obtained such dissociated and spread sea urchin sperm

chromatin for electron microscopy by first treating the cells with the chelating agent EDTA and then by spreading them on a water surface (15). Brooke, et al. uncoiled and dissociated human metaphase chromosomes by placing cells in a hypotonic potassium chloride (KCl) solution which did not contain divalent cations (73).

The binding sites of Ca^{++} and Mg^{++} in calf thymus nuclei were studied by Naora, et al. (Table 1). Of the total nuclear Mg^{++}, 64% was bound tightly to phosphate groups, primarily on DNA but also on RNA and mononucleotides; and of the total DNA phosphate groups, one-tenth were available to Mg^{++} for binding and nine-tenths were neutralized by histone. Twenty nine per cent of the intranuclear Mg^{++} was soluble in the isolating medium, and 7% was soluble in the incubating medium. Only 24% of total nuclear Ca^{++} was firmly bound, and this portion was apparently associated with proteins rather than DNA, RNA, or mononucleotides. A second portion of Ca^{++}, 58%, which may be attached to nucleic acids was soluble in the isolation medium; and a third portion, 18% was soluble in the incubation medium (39).

Indirect and direct evidence that Ca^{++} and Mg^{++} are structural components of chromosomes has been presented by Steffensen. He found that culturing Trandescantia in water without Ca^{++} or Mg^{++} readily caused chromosome fragmentation (20 to 30 times the control rate of fragmentation); this effect was promptly reversed upon addition of Ca^{++} to normal levels. Direct evidence that Ca^{++} occurs in chromosomes in a stable linkage was obtained from autoradiographs of radioactive Ca^{++}($^{45}Ca^{++}$). After germination of radioactive Easter lily pollen on non-radioactive flowers and after one subsequent mitotic division he observed labeled sperm nuclei and tube nuclei. Additional experiments involving meiotic division in the parasitic wasp Habrobracon revealed that sperm of adult male wasps that had been grown from the time of fertilization in a $^{45}Ca^{++}$ environment contained radioactive concentrations in the nucleus

(69,70). Naora, et al have suggested that this stable Ca^{++} may be comparable to the stable Ca^{++} which they observed in calf thymus nuclei (39).

Although RNA is found in interphase and metaphase chromosomes (Table 1), very little data pertaining to this association is available. It is not likely that this RNA is essential for maintaining chromosome and chromosome fiber structure, for RNAase treatment causes no alteration of these structures (13). Frenster has found RNA molecules which are de-repressors of RNA synthesis; he has also found greater amounts of RNA in active chromatin than in inactive chromatin (67).

To recapitulate briefly, in this section the chromosomal components DNA, histones, non-histone proteins, lipids, cations, and RNA have been discussed with respect to their individual properties and their mutual interactions.

Structural organization of chromosomes

Chromosomes of eukaryote species, i.e., higher organisms, are complex supramolecular systems which efficiently maintain and transport genetic information. The structural organization of chromosomal components into such systems has been a subject of investigation for many years, but many questions remain unanswered. This review will deal with the fundamental requirements of a chromosome model, with genetic, cytologic, irradiation, and electron microscopic data which relate to such requirements, and with possible models of chromosome structure. A number of comprehensive reviews of chromosome structure are available (5,11,69,72).

A chromosome model must meet certain fundamental criteria. Provision for packaging a large amount of DNA with associated histones, non-histone proteins, cations, RNA, and lipids is required; at times portions of such a package must be partially extended (the euchromatin of interphase nuclei) and at other times must be very compact and tightly coiled

(the heterochromatin of interphase nuclei; prophase, metaphase, and anaphase chromosomes). In addition this package must replicate, as does DNA, in a semiconservative manner; and it must allow mechanisms for mutation, recombination, breakage and reunion, and translocation.

A consideration of the amount of DNA in individual chromosomes gives additional perspective to the packaging problem. A diploid human lymphocyte nucleus contains 6×10^{-12} gm of DNA (Davison & Osgood, quoted in (75)). An average molecular weight of DNA is 8×10^6 (47), and from Avogadro's number such a molecule is calculated to weigh 1.33×10^{-17} gm. The number of DNA molecules per diploid (anaphase) cell is $6 \times 10^{-12} / 1.33 \times 10^{-17}$, or 4.5×10^5. If a DNA molecule in an uninterrupted Watson-Crick configuration is 4 μ in length (46), the total length of DNA per diploid cell is $4 \times 4.5 \times 10^5 = 180$ cm; and the average length of DNA per anaphase chromosome is 180/46 = 3.9 cm. DNA is associated with proteins, and Zubay and Doty have estimated that the linear length of the deoxyribonucleoprotein (DNP) complex is about one-half the linear length of DNA (46). Therefore, the average total linear length of DNP in an anaphase chromosome approximates 2 cm. This must be packaged into a cylinder (an average anaphase chromosome) which is 4.8 μ long (10) and 1 μ in diameter (16) and which has a volume of 3.8 μ^3.

These considerations provoke a number of questions. What is the composition, diameter, length, and three dimensional arrangement of the primary chromosome fiber? Does such a fiber extend the length of a chromosome? How does the primary fiber replicate and how does this relate to semiconservative chromosome replication? Does a chromosome contain one (unineme) or more than one (polyneme) primary fiber? Experiments which have dealt with these problems will be reviewed in the following paragraphs.

In terms of genetic phenomena such as dominance, mutation, recombination, breakage and reunion, and translocation, a single-stranded chromosome is most acceptable. In fact, a major argument against the polyneme concept is the difficulty of constructing a model which accounts for these genetic occurrences. Two recent papers are of interest in this respect. In 1965 Uhl proposed a new theoretical model for classical intergenic crossing over which was based on structural and replication properties of chromosomes. He suggested that semiconservative replication of chromosomes may occur if single (unineme) or multiple parallel (polyneme) DNA double helices are linked end to end by molecules (probably residual protein) which function as single units during the assortment processes of meiosis and mitosis (76). Maguire observed chromatid doubleness at first meiotic prophase in maize and proposed a bineme chromatid model in which crossing over precedes replication and is followed by semiconservative distribution (77).

A major portion of the argument for multistrandedness in chromosomes has come from cytologic observations. Manton in 1945 described double stranded anaphase chromosomes in the fern Todea (78). More recently Peacock has published photographs which illustrate half-chromatids in anaphase chromosomes of Vicia faba (79). Half-chromatids have also been seen and photographed in living anaphase cells of Haemanthus (Bajer, quoted in (79)). Chromatid subunits become more apparent after experimental alteration of chromosomes. Trosko and Wolff demonstrated Feulgen positive half-chromatids and possible quarter-chromatids in isolated metaphase chromosomes of Vicia faba after exposure to trypsin (80). Brooke, et al. observed half and likely quarter-chromatids in human metaphase

chromosomes which had been uncoiled and dissociated in 0.01 M KCl (73). Contrary to the implications of the preceeding experiments, Gall concluded from the kinetics of DNAase action on newt oocyte lampbrush chromosomes that the lateral loops were single stranded (unineme) (81).

The use of tritiated thymidine (^{3}HT) and autoradiography has provided much insight into the cell cycle, the mechanism of DNA replication and segregation, and the length of replicating DNA molecules. In 1957 Taylor, Woods, and Hughes by employing ^{3}HT for autoradiography demonstrated semiconservative replication of Vicia faba chromosomes (82). Similar studies in many other eukaryote species have documented semiconservative replication of chromosomes so well that there is no doubt that this is the mechanism of DNA and chromosome duplication. This data is most readily explained by the single-stranded chromosome concept. However, Peacock has observed isolabeling of chromatids at the second division (X_2) after incorporation of ^{3}HT. Although this phenomenon occurs rarely, it is interpreted to mean that subchromatid exchanges took place and therefore that the chromatid is at least a bineme structure (83). Recent applications of Cairns' ^{3}HT autoradiography technique to chromosomes of eukaryotes have provided estimates of the replicating DNA fiber length and configuration. Cairns by labeling HeLa cell DNA with ^{3}HT saw lengths of DNA that were at least 500 μ long. After determining that the human DNA duplication rate was 0.5 μ per minute or less, he suggested that 100 or more duplication sites exist in each chromosome and that these sites are linked in one long series (84). Huberman and Riggs performed similar experiments with Chinese hamster cells and observed that 6% of the DNA autoradiographs were 800 μ long and that rare DNA autoradiographs were 1.6 to 1.8 mm long (85). Neither of these articles reported circular

DNA fiber configurations such as those found in bacteria and viruses.

Radiation of cells has been employed to study chromosome breakage and reunion and degrees of strandedness. Wolff has found in Vicia faba chromosomes that protein, but not DNA synthesis is necessary to effect repair of radiation induced breaks. In other experiments he has combined irradiation with ^{3}HT labeling of chromosomes. He observed chromosome aberrations in most of G_1 (pre-DNA synthesis period) and chromatid aberrations in the last two hours of G_1, in very early S (DNA synthesis period), and in G_2 (post-DNA synthesis period). These findings were interpreted to mean that the chromosome was at least two stranded (polyneme), i.e., two Watson-Crick molecules, in G_1 (86). Moses has offered an alternative explanation which contends that the anaphase chromosome contains a single Watson-Crick molecule (unineme) and that separation of sister DNA helices prior to replication allows radiation damage to only one chain which subsequently appears as a chromatid break (11).

The electron microscope has added much to our knowledge of chromosomes. A number of investigations of chromosome ultrastructure are summarized in Table 2. Two methods have been employed for preparing chromosomes for microscopy: thin sectioning fixed and embedded cells and placing cells, nuclei, or metaphase chromosomes directly on carbon coated grids as whole mounts. In general, electron micrographs of ultrathin sections of chromosomes reveal little more than granules and short fibrils; but this is of value because it provides partial confirmation of results from whole mount preparations. DeRobertis in 1956 described 47 Å fibers in early prophase, 70 Å fibers in late prophase, and 100 Å fibers in metaphase chromosomes of thin sectioned primary spermatocytes of the grasshopper (1). Fawcett's

micrographs are typical of most thin sectioned chromosomes in that they reveal little more than 100 Å granules (2). Electron microscopic autoradiography of thin sectioned materials has yielded significant information. Hay and Revel studied nuclei and chromosomes of regenerating salamander limbs and observed that the nucleoprotein was a meshwork of interconnected filaments 50 to 75 Å in diameter. The meshwork was both dense and dispersed in interphase nuclei but was only dense in metaphase chromosomes. By electron microscopic autoradiography they determined that DNA synthesis occurred later in dense than in dispersed chromatin of interphase nuclei (8). By using tritiated uridine and electronmicroscopic autoradiography Littau, et al. demonstrated that RNA synthesis in calf thymus nuclei occurs in areas where 100 Å chromosome fibers are dispersed and loosely arranged (87). The 100 Å fibers of such areas (euchromatin) are continuous with fibers in densely packed areas (heterochromatin) where RNA synthesis does not occur (88).

Whole mount preparations of chromosomal material are the most rewarding means of studying chromosome structure with the electron microscope. Usually whole cells, isolated chromosomes, nuclei, or nucleohistones are placed directly on carbon coated grids or are spread and dissociated on a water surface. The specimen remains adherent to the grid which then serves as a vehicle for various experimental procedures, staining, dehydration, and drying. Either positive or negative staining is employed. Negatively stained materials are dried in air, and positively stained materials are dried by the critical point method (7,9,12,13,15,89,90).

Early experiments did not employ such spreading and drying techniques,

and consequently the resolution was less than that achieved in the past four to five years. In 1956 Gall used replica techniques for preparing oocyte lampbrush chromosomes of Triturus. He observed chromosome fibers which were single, very long and irregular, and 500 Å in diameter and which were not disrupted by pepsin (3). At about this same time Ris began examining whole mount preparations of lampbrush chromosomes which were either shadowed or dried by the Anderson critical point method. He observed 500 Å coiled chromosome fibers in Triturus and Necturus (91). In 1961 and 1962 he reported observations of 200 Å coiled fibers in whole mount preparations of lampbrush chromosomes of Triturus viridescens and of leptotene chromosomes of Tradescantia and Lilium. Thin sections of Tradescantia and Lilium chromosomes revealed 100 Å "rodlets" which were termed the "elementary chromosome fibril." The next level of organization was seen in calf thymus nucleohistone which was isolated in saline-versene at pH8 or which was exposed to 0.2N HCl for extraction of histones; the 100 Å fiber was composed of two 40 Å fibers. The 40 Å fibers were interpreted to be single nucleohistone molecules which suggested that the 100 Å fibers were composed of two DNA double helices with associated histones. Fibers of 40 Å diameter were also seen in sperm from many species (5,6). In 1963 Ris and Chandler reported that erythrocytes of T. viridescens which were spread on a water surface contained 250 Å fibers that had two 100 Å subunits. The 100 Å fibers in turn were composed of two 40 Å fibers; after treatment of such preparations with 0.2N HCl 20 to 40 Å fibers were observed. The organizational pattern seemed to be pairing of pairs, and Ris concluded that the chromosomes of eukaryotes were multiple stranded, consisting of at least two and generally more subunits (9).

In 1963 in an article on Triturus erythrocyte chromosome fibers Gall

introduced the water surface method of spreading chromosome fibers. He observed long 400 to 600 Å fibers which exhibited very few breaks where there was little tension. These and his previous observations aligned Gall with the unineme concept of chromosome structure (7). In 1964 Osgood, et al. reported the second electron microscopic study of whole mount human metaphase chromosomes. The first whole mount investigation of human chromosomes by Barnicot and Huxley revealed little more than was seen with the light microscope (4). Osgood's studies of chromosomes which were partially dissociated, fixed in acetic alcohol, and air dried revealed apparent multiple 500 Å strands in each chromatid; and he proposed a "rope" model which was similar to that of Steffenson (70) and which contained 16 DNA molecules per chromatid (10).

Within the past year there has been much activity in the area of whole mount electron microscopy of chromosomes. DuPraw observed long fibers of 230 to 250 Å diameter in both interphase and metaphase cells of honey bee embryos. These fibers were folded and twisted in helices, and a 30 to 50 Å core was present after trypsin treatment. This core was disrupted by DNAase (12). In a subsequent paper he proposed a 'folded fibre' chromosome model in which each chromatid contained a single Watson-Crick DNA molecule (larger chromosomes possibly contained many DNA molecules linked end to end in series) with associated proteins (histones and acidic proteins). These fibers were packaged into the chromosome by folding (16). His next paper dealt with whole mount, critical point dried human lymphocyte metaphase chromosomes; he observed interphase fibers of 230 Å diameter and metaphase fibers of 300 Å diameter. His

observations also included parallel fibers within a chromatid which were interpreted to be consistent with a slightly modified 'folded fibre' model (17). Wolfe employed similar techniques in studying T. viridescens and T. granulosa nucleated erythrocytes and cultured bovine kidney metaphase chromosomes. These chromosome fibers were 250 Å in diameter, coiled, and occasionally paired. If the cells were spread on the surface of a 1.0M NaCl solution he observed fibers ranging from 20 to 30 Å to 250 to 300 Å in diameter. Treatment with RNAase did not affect the fibers, but DNAase treatment disrupted them (13,14). Solari investigated the structure of sea urchin sperm chromatin. He employed EDTA and a water surface for spreading, and then used uranyl acetate for negative staining. He observed 40 Å fibers which were coiled and frequently paired with another 40 Å fiber. These fibers were disrupted by DNAase and were made smaller and smoother by trypsin. Following extraction of histones (sea urchin sperm contains typical histone and non-histone protein (5)) with 2.0M NaCl he observed 20 Å fibers. When chymotrypsin was complexed with DNA he saw few free ends, and he was able to trace some chromosome fibers for lengths of 50 to 93 μ without interruption.

A recent paper by Hotta and Bassel contains electron micrographs of isolated boar sperm DNA in circular configurations; the circumference of the circles was 0.5 to 9.7 μ for small circles and up to 16.8 μ for large circles. A large number of non-circular fibers were also observed (92).

To conclude this section, chromosome models will be considered briefly. It is apparent that there are two distinct concepts of chromosome structure: the unineme model and the polyneme model. As much as possible the exper-

imental data has been presented in an unbiased manner. Perhaps this makes the story more complex and confusing, but this is the state of affairs at this time.

The proponents of the unineme model contend that an anaphase chromosome consists of a single nucleoprotein fiber (11,16,17,74). DNA molecules maintain the longitudinal continuity of the fiber, are associated with histones, may extend the length of the chromosome, or may be interspersed by linkers which join molecules in series. This fiber becomes compact by folding and coiling. Those who believe in the polyneme concept hold that a chromatid contains two (bineme) or more nucleoprotein fibers (5,9,10,69,70,79,80,86). The multiple fibers are envisioned to be parallel, associated by pairs, or in a rope-like configuration (10,70).

MATERIALS AND METHODS

Tissue culture

Lymphocytes from healthy individuals were cultured at 37°C in 10 ml of Medium NCTC 109 (Microbiological Associates Inc., Bethesda, Md.) which contained 10% human serum (Hyland Laboratories, Los Angeles, Calif.), 100 units/ml penicillin G, and 0.05 cc/ml phytohemagglutinin (1:12 dilution of crude extract (93)). Cells at interphase were obtained from cultures harvested after 48 hours and 72 hours; cells at metaphase were obtained by adding colcemide (0.2 µg/ml) at 60 hours and harvesting cultures at 72 hours.

Lymphocyte strain M166 which was derived by adding blood from a patient with chronic granulocytic leukemia to cell strain Oregon J111 (94) was maintained in continuous culture in Minimum Essential Medium-Eagle, Earle's base (Baltimore Biological Associates, Baltimore, Md.), at 37°C. Metaphase cells were obtained by adding colcemide (0.2 µg/ml for 12 hours) or vincoleukoblastine (0.5 µg/ml for 12 to 24 hours) to cultures which 48 hours previously had received fresh media.

Whole mount preparation

Lymphocytes in primary culture were harvested by withdrawing 8 ml of medium from the culture vials and suspending the cells in the remaining 2 ml of medium by swirling and gently agitating with a Pasteur pipette. This suspension was placed in a 15 ml graduated centrifuge tube, and the culture vials were rinsed with culture medium

which subsequently was added to the centrifuge tube. M166 lymphocytes were harvested by vigorously swirling the culture medium which dislodged cells in mitosis from the glass surface and then by decanting the suspension into 15 ml centrifuge tubes. After centrifugation at 1000 to 1200 rpm for 10 minutes a loose "button" of cells was present, and all but 0.1 to 0.2 ml of the supernatant media was discarded. The pellet of cells was resuspended by gentle agitation with a Pasteur pipette, and about 0.1 ml of concentrated cells and 0.9 ml of 0.01M KCl at 42°C was drawn into a disposable Pasteur pipette. The pipette, with a 2 ml suction bulb in place, was closed with 'Plasticine' modelling clay (the cell suspension was not allowed to touch the clay) and was placed vertically in an incubator at 42°C for one-half to three hours. Following this, four to five drops of the cell suspension were placed directly on Formvar-carbon or Parlodion-carbon coated 100 or 200 mesh athene or regular copper grids. After each drop the excess fluid was drawn off gently with filter paper without allowing the grids to dry, and usually four to five cells remained adherent to the supporting membrane. Spread and dissociated chromosome fibers also were obtained when 0.1M potassium or sodium ethylenediaminetetraacetic acid (60 minutes, room temp.) or 0.3% trypsin (2x crystallized, adjusted to pH 8.0 with 0.01N NaOH, 37°C, 60 to 90 minutes) was substituted for 0.01M KCl.

Enzymatic treatment

Enzymatic experiments were carried out by floating the grids cell side down on 5 to 10 drops of one of the following solutions prior to

staining: DNAase 1x crystallized, 2 mg/ml, MgCl 1.2 mg/ml, adjusted to pH 6.5 with 0.01N NaOH, 37°C, 20 minutes (6, 12, 13, 15, 81); RNAase crystallized from ethanol, 0.5 mg/ml, adjusted to pH 7.0 with 0.01N NaOH, 37°C, 60 minutes (13); trypsin 2x crystallized, 1 to 3 mg/ml, adjusted to pH 8.0 with 0.01N NaOH, 37°C, 25 to 60 minutes (12,15); alpha-chymotrypsin 3x crystallized, 0.4 mg/ml, pH 6.5, 37°C, 30 to 60 minutes (15). All enzymes were obtained from Worthington Biochemical Corp., Freehold, N. J.

Salt and/or acid treatment

Salt and/or acid experiments were carried out by floating the grids cell side down on one of the following solutions prior to staining: NaCl, 2.0M, 20 to 180 minutes, room temperature (15,62,95); HCl, 0.2N, 60 minutes, room temperature (9,48); citric acid 0.1M-NaCl 0.125M, pH 2.0, 60 minutes, room temperature, followed by HCl, 0.2N, 60 minutes, room temperature (96).

Staining

The cells were stained positively by floating the grids cell side down on uranyl acetate (2.0%, pH adjusted to 4.5 with stock veronal acetate buffer, 5 to 10 minutes) (14,97) or phosphotungstic acid (1.0%, pH adjusted to 4.5 with 1.0N NaOH, 10 to 15 minutes) (15).

Drying the preparations

After thorough rinsing in water to remove excess stain the grids were placed in a carrier and then were dehydrated in ethanol (35,50,70, 95,100,100,100%; 5 minutes each), passed through amyl acetate

(1:1 amyl acetate:absolute alcohol, 100%, 100% amyl acetate; five minutes each), and placed in the pressure chamber of the Anderson critical point drying apparatus (89). After assembly of the apparatus the pressure chamber was immersed in ice water (10 to 15°C) and was filled with liquid CO_2. The exhaust valve was slightly opened for 10 to 15 minutes which allowed liquid CO_2 to flow through the vessel to replace the amyl acetate. All valves were closed and the vessel was submerged in a 45°C water bath to raise the temperature within the pressure chamber. Drying occurred instantly and with little distortion when the temperature was raised above 31°C (the critical point for CO_2 when there is equilibrium between the gas and liquid phases). When the pressure stabilized (usually about 1500 - 1700 psi) the exhaust valve was opened slightly to release gaseous CO_2 and the vessel was allowed to return slowly to atmospheric pressure over a 10 minute period (12, 13, 90).

Thin section preparation

The pellet of cells obtained from centrifugation was broken into smaller clumps of cells. Cells were fixed in either glutaraldehyde (3% in 0.1M phosphate buffer, pH 7.2) (98) or OsO_4 (1% containing 0.1% $CaCl_2$ and 0.1% tryptone pH 6.2) (99,100). After fixation cells were stained with 0.5% uranyl acetate (pH adjusted to 5.0 with stock veronal acetate buffer) (99). Dehydration in alcohol and propylene oxide and embedding in Epon 812 were done by standard methods (101). Thin sections were obtained with a Porter Blum Model MT-1 microtome.

Electron microscopy

The preparations were photographed with an RCA model EMU-3F electron microscope. For the whole mounts the microscope was used at 100KV with a 250 micron condenser aperture and 25 to 45 micron objective apertures. For the sectioned cells 50 KV was used with the same apertures. Astigmatism was corrected to 0.25 μ to 0.50 μ. After normalization of the lenses magnification was calibrated with 54, 864 and 28,800 lines per inch carbon replica gratings (Ladd Research Ind., Inc., Burlington, Vt.). Kodak Projector slide plates (contrast) were employed for all photography.

Measurements

All measurements for magnification calibration and for fiber diameter determinations were made directly on the negative plate with a Gaertner toolmaker's microscope (Gaertner Scientific Corp., Chicago, Ill.). Chromosome fibers were selected for measurement if they were uncoiled (straight, uniform diameter), distinct, and the smallest fibers of the preparation. By selecting only uncoiled fibers for measurement excessive variance of individual fiber diameters due to coiling was avoided. Three diameters were determined at 250Å intervals along each fiber.

RESULTS

The data on chromosomes and chromosome fibers were obtained from phytohemagglutinin stimulated normal human lymphocyte cultures. A summary of the experiments from which these data were collected is presented in the Appendix. Cells from stock cultures of strain M166 were employed in preliminary stages of this investigation, but data obtained from these cells is not included in this thesis with exception of the micrographs of thin sectioned nuclei and chromosomes (Figures 14-16).

Suspending cells in hypotonic KCl and then placing the suspension dropwise on a carbon coated grid was an effective method for securing well spread chromosome fibers from cells at any stage of mitosis. This method was also effective when ethylenediaminetetraacetic acid (EDTA) or trypsin was substituted for KCl. With the cells adherent to the grid staining or experimental procedures prior to staining were carried out easily by placing the grid cell side down on the desired solutions. Dehydration in alcohol followed by drying in the critical point apparatus yielded preparations which were superior to those preparations dried in air.

General characteristics of chromosome fibers

In whole mount preparations interphase and metaphase chromosome fibers which had been spread and dissociated in 0.01M KCl had a similar appearance. They were long, often extending 5 to 15 micra without interruption (Figures 1,4,8,11), and they exhibited varying degrees of coiling (Figures 2,3,5,7,12). Uncoiled fibers were selected for measurements

because large variations in chromosome fiber diameter were associated with coiling (Figures 2,3,5,7,12). From these measurements a histogram of the relative frequency of uncoiled fiber diameters was constructed, and two peaks were observed. The calculated means and standard deviations of these two populations were 66±12 Å and 112±18 Å, and the observed distributions approximated normal distributions (Figure 13). The 66 Å fibers were seen both singly and associated closely with another fiber of similar size; in the latter configuration the overall diameter of the pair often fell within the range of the 112 Å group (Figures 12, 47). The 112 Å fibers sometimes were divided longitudinally into two subunits each of which had diameters of about 66 Å (Figures 12,47).

Thin section preparations of fixed and embedded intact cells at interphase, metaphase, and telophase provided little information about chromosome structure. At each of these mitotic stages sectioned chromosomes appeared as masses of granules and short fibers with diameters that fell within the 112 Å population (Figures 14-16).

Structural roles of chromosome fiber components

To define the structural roles of the molecules and macromolecules of chromosome fibers the cells were treated with salts, chelating agents, enzymes, and acids. The results of these experiments are presented in Table 3. Potassium chloride (0.01M) and sodium or potassium EDTA (0.1M) readily produced spreading and dissociation of chromosome fibers (Figures 1, 4,11,17,18); however at these concentrations EDTA usually produced less dissociation and uncoiling than KCl (Figures 1,19). Longer exposure of cells to hypotonic KCl increased the degree of dissociation, particularly for interphase cells.

For experiments which were designed to characterize the structural roles of chromosome fiber components other than divalent cations, hypotonic KCl was employed to obtain spread and dissociated fibers. The longitudinal continuity of dissociated chromosome fibers was disrupted by DNAase: no uncoiled fibers comparable in length to those of untreated cells were seen. The fibers which remained after DNAase treatment were short fragments of uncoiled fibers and longer segments of highly aggregated and coiled fibers. These fibers generally appeared less well organized than those of untreated cells (Figures 22,23). Exposure of dissociated cells to RNAase produced no detectable difference in fiber morphology (Figures 24-26). Following treatment with trypsin, dissociated chromosome fibers exhibited less coiling but exhibited no disruption of longitudinal continuity or decrease in fiber diameter (Figures 27,28). When trypsin was substituted for 0.01M KCl only partial spreading and uncoiling occurred, and this revealed folding and coiling patterns of 112 Å chromosome fibers (Figures 29-33). Chymotrypsin produced a change in the coiling pattern of dissociated fibers but did not break them (Figures 34,35). Exposure of dissociated fibers to 2.0M NaCl produced more uniform uncoiling and increased the number of smaller fibers: after 20 minutes an increased proportion of fibers within the 66 Å population was present (Figure 37), and after 60 minutes a third fiber population with an uncoiled fiber diameter of 37 ± 6 Å was defined. The 37 Å fibers were seen both singly and associated with another fiber of similar size (Figures 13,39-42). Observations of lesser degrees of coiling and smaller fibers were not constant with NaCl treatment (Figure 38), but such observations were never made in preparations that were dissociated, stained, and dried in the usual manner. Subjecting

dissociated fibers to 0.2N hydrochloric acid produced increased numbers of fibers in the 66 Å population (Figure 44), but did not break them. (Figures 43,44). Citric acid (0.1M) and NaCl (0.125M) followed by 0.2N HCl produced less coiling and 37 Å fibers which were sometimes paired. This treatment did not disrupt the longitudinal continuity of the fibers (Figures 45,46).

Organizational patterns of chromosome fibers

In whole mount preparations masses of chromosome fibers originated from relatively compact and well packaged structures: nuclei (Figures 1, 2,24,36,38) and metaphase chromosomes (Figures 8-12). Some patterns of chromosome fiber packaging were observed (Table 3). Close association of two fibers of the 66 Å population (Figures 12,47) or of the 37 Å population (Figures 41,42,46,47) was seen, and these fibers often formed a single larger fiber. Chromosome fibers were coiled to some degree in all preparations, and coiling readily condensed the fibers and increased their apparent size (Figures 3,19,20,21). Folding, coiling, and looping of the fibers was seen following comparatively mild uncoiling and dissociation, particularly when trypsin (Figures 29-33) or EDTA (Figures 19-21) was substituted for KCl; but these patterns were seen also in some cells treated with hypotonic KCl (Figure 7).

DISCUSSION

In the early stages of this study chromosomes were examined in ultrathin sections, but this method proved to be uninformative. A means of securing whole mount preparations was needed; a new, relatively simple approach was suggested by the observation that hypotonic KCl causes dissociation and uncoiling of human metaphase chromosomes (73). Indeed, well spread and dissociated preparations were obtained by suspending cells in 0.01M KCl and by subsequently placing the suspension directly on carbon coated grids. This technique was also successful when cells were suspended in EDTA or trypsin; thus, three different means of securing whole mounts of chromosomes and chromosome fibers were available. Electron micrographs which were obtained of these preparations permit ultrastructural characterization of human chromosome fibers and give some insight into the organizational patterns of these fibers within chromosomes.

The materials and methods which were employed in this investigation in some respects are unique. There has been only one other published electron microscopic study of normal human lymphocyte chromosomes in which the combination of the whole mount technique and critical point drying was employed (17). To this author's knowledge there have been no other ultrastructural studies of chromosomes which have utilized hypotonic KCl or trypsin in combination with critical point drying.

Two aspects of the methods deserve further discussion. Dissociation and spreading of chromosomes is probably related to two factors. First, placing cells in suspension in 0.01M KCl, EDTA, or trypsin alters the chromosome structure; and second, placing a drop of the cell suspension

on the grid membrane followed by removal of excess fluid with filter paper probably causes dissociating and spreading forces. Critical point drying is essential for good preservation of fiber morphology. Drying preparations in air causes undue distortion of the fibers, primarily from strong forces at air-water interfaces as drying occurs; as a result specimens appear flattened onto the membrane surface. By contrast, critical point drying, i.e., instant drying with minimal distortion at 31.1°C, preserves three dimensional configurations, coiling, looping, and folding of fibers.

The chromosome fiber

This section will deal with the characteristics and the components of the chromosome fiber. Because no significant difference was observed between dissociated and spread fibers of interphase and metaphase cells, the term "chromosome fiber" will be employed in a general way and will not imply a specific time in the mitotic cycle. Fiber length and the determinants of longitudinal continuity and fiber diameter and the determinants of diameter will be discussed.

Dissociated and spread chromosome fibers were often observed to extend 5 to 15 μ without interruption (Figures 1,4,8,11). This is not a surprisingly long distance when it is considered that the theoretical linear length of deoxyribonucleoprotein (DNP) in an anaphase chromosome is 2 cm. It is even a less surprising distance when compared to the 90 μ long chromatin fibers in sea urchin sperm (15) which Solari observed with the electron microscope, to the 0.5 mm long fibers of duplicating DNA in HeLa cells (84), and to the 1.6 to 1.8 mm long fibers of duplicating DNA

in Chinese hamster cells (85). The factors which maintain the longitudinal continuity of this fiber were examined.

Exposure of dissociated and uncoiled chromosome fibers to DNAase caused disruption of their longitudinal continuity (Figures 22,23). Du Praw (12,16), Wolfe (13,14), and Solari (15) have reported the same observations in a number of different species (Table 2). This indicates that fiber integrity depends upon intact DNA macromolecules. The presence of only short segments of uncoiled chromosome fibers after DNAase treatment indicates that there are numerous areas on the dissociated uncoiled fiber where phosphate-3'deoxyribose bonds are accessible to the DNAase. This implies that in the uncoiled fibers histones or other proteins do not form a thick covering over DNA. On the contrary, coiled and tightly aggregated fibers remain relatively intact following DNAase exposure; thus there must be fewer vulnerable bonds.

The long fibers which were observed in this study and the exceedingly long fibers which were observed in other studies require evaluation in terms of DNA molecular weight and linear length and of end to end linkers between DNA molecules. Linkers joining DNA molecules end to end are not likely to be detected on a morphologic basis even with the electron microscope; and they certainly would not be recognized by light microscopic autoradiography which has only 1 μ resolution. If linkers are present, experiments involving their enzymatic degradation or their extraction might provide detectable fiber breaks or shorter fibers. In this investigation treatment of uncoiled chromosome fibers with trypsin, chymotrypsin, RNAase, NaCl, HCl, citric acid-NaCl, or EDTA

caused no observable alteration in fiber longitudinal continuity; this indicates that there probably are no linkers joining DNA molecules end to end at intervals of less than 5 to 15 μ. Similar observations have been reported by Solari for NaCl, trypsin, chymotrypsin, and EDTA in sea urchin sperm chromatin (15); by Wolfe for RNAase in amphibian and bovine chromosomes (13,14); by Du Praw for trypsin in honey bee chromosomes (12); by Huberman and Riggs for pronase in Chinese hamster DNA fibers (85); and by Hotta and Bassel for RNAase and pronase in wheat DNA fibers (92). Thus, if linkers are present they are seemingly unaffected by a wide range of agents. However, the possibility that bonds which are critical to maintenance of linker integrity are not accessible or sensitive to these agents should be considered. Thioglycol as suggested by Dounce may be useful in detecting linkers if they depend on disulfide bonds for integrity (58). It seems quite possible for two reasons that DNA is actually larger than the usually given molecular weight of 8×10^6 and the corresponding length of 4 μ. First, DNA is easily degraded by even mild shearing forces (47). Second, Cairns has estimated that the average mammalian chromosome which contains about 3 cm of DNA has at least 100 sites of DNA duplication. If it is assumed that a duplicating unit of DNA is one DNA molecule, then 30,000/100 = 300 μ as the length per DNA molecule (one duplicating unit).

Chromosome fiber diameter has been a difficult parameter to evaluate because coiling of fibers increases the diameter and also causes a large variance of individual fiber diameter. For these reasons only uncoiled fibers (straight, uniform diameter) with diameters less than 150 to 175 Å were measured. Three populations of fibers from

interphase and metaphase chromosomes may be defined by uncoiled fiber diameter (Figure 13).

Fibers of the population with the smallest diameters, 37±6 Å, are observed only after treatment of dissociated cells with 2.0M NaCl (Figures 40-42) or with 0.1M citric acid-0.125M NaCl followed by 0.2N HCl (Figures 45,46). Each of these experimental procedures is expected to extract histones from chromosome fibers, and the resulting fiber diameters confirm this expectation. The 37 Å fibers are composed primarily of a single DNA double helix; the smaller fibers of this population agree with x-ray diffraction and electron microscopic determinations of the DNA fiber diameter (42), and the larger fibers of this group may be partially coiled and may have some associated protein. While it is possible that the 37 Å fiber is composed of two DNA double helices, this does not seem likely because this population has a normal rather than a bimodal distribution. Similar results were obtained by Solari (15), by Wolfe (13,14), and by Ris (9) (Table 2). The 37 Å fibers were sometimes observed to be paired, and this will be discussed in following paragraphs.

The populations of intermediate fiber diameter and large fiber diameter are observed after dissociation with KCl. Because they overlap, each population was defined by assigning distribution tails in the overlap area similar to their tails at the extremes. Thus, values of 66±12 Å and 112±18 Å were calculated. The 66 Å fiber is the smallest fiber seen in chromosomes which are dissociated with KCl. If such dissociated chromosomes are exposed to 2.0M NaCl for 20 minutes or 0.2N HCl for 60 minutes, 66 Å fibers both singly and in pairs are more frequently observed.

Apparently treatment with these agents extracts molecules which are important in cross linking 66 Å fibers. It may be speculated that these molecules are lysine rich histones, for this is the first histone fraction to be extracted by NaCl or HCl (48). Further support is given this speculation by the paper of Littau, et al. which reports the differential extraction of lysine rich histones in calf thymus nuclei with subsequent separation and loosening of the chromatin network (87).

The 112 Å fiber is the most frequently seen uncoiled chromosome fiber in preparations which are dissociated with KCl. Coiling increases its diameter to 200 to 300 Å. In the uncoiled state it is sometimes seen to be composed of two smaller fibers, each of which falls within the 66 Å population. This fiber is altered by procedures which alter protein molecules. There are increased numbers of smaller fibers which are within the 66 Å and 37 Å populations and which are sometimes paired after NaCl, HCl, or citric acid-NaCl-HCl treatment. As stated previously, this is taken as evidence that partial removal of proteins decreases cross linking forces. After trypsin treatment there is decreased coiling, but there is no segmental or overall change in the fiber diameter (Figures 27,28). The reason larger segments of protein were not removed by trypsin is not altogether clear; but probably the protein was not in an optimum condition for trypsin action. That is, the protein was not denatured and arginine-lysine bonds were not readily accessible to the enzyme. Chymotrypsin produced a previously unseen configuration of the 112 Å fiber. It appeared that short segments of uncoiled fiber (500 - 2000 Å) alternated with shorter regions of coiling (Figures 34,35). This phenomenon of "coil-uncoil" was a constant finding in three cells which were examined.

Organizational patterns of chromosome fibers

In this section the chromosomal components will be related to observed organizational patterns which result in packaging of chromosome fibers. These patterns include coiling, folding, looping, and pairing of chromosome fibers.

Coiling which was seen in nearly all preparations is the most general mechanism of chromosome fiber packaging. The degree of coiling is influenced by the ionic environment and by the proteins associated with DNA. Removal or dilution of Ca^{++} and Mg^{++} with EDTA or KCl reduces the extent of coiling (Figures 1,17), however EDTA generally produces less uncoiling than KCl (Figures 19,20). The partially dissociated interphase cell in Figures 19-21 illustrates packaging and condensation of apparently continuous long chromosome fibers by coiling, folding, and looping. Because about one-tenth of the DNA phosphate groups are not neutralized by histones, Mg^{++} and Ca^{++} interact with these groups; and in addition, Ca^{++} is probably firmly bound to the chromosomal proteins (39,69). Such interactions probably result in chromosome fiber coiling. Coiling is also due to proteins. Extraction of histones with salt, acid, or enzymes decreases the coiling. The proteins probably influence coiling of DNA by neutralizing the negatively charged phosphate and by cross linking the DNA backbone. Double helical DNA remains intact at a radius of curvature as small as 400 Å (46). The relative importance of divalent cations and proteins in maintaining coiling is not well understood. However in these experiments KCl, which probably displaces and dilutes chromosomal Ca^{++} and Mg^{++}, caused a greater extent of uncoiling than did trypsin which alters the chromosomal

proteins (Figures 1,4,29-33). Trypsin is a relatively gentle means of dissociating chromosome fibers, and their organizational patterns often remain relatively intact (Figures 29-33). In the cell of Figures 29-33 the chromosomal material appears to be a continuous long fiber (112 Å diameter at one point) which is first coiled and folded and is then looped upon itself. The looping and gross spiralling strikingly resembles chromatid coiling as seen with the light microscope; and the organizational patterns of this chromosomal material could be similar to the organization of chromosome fibers within metaphase chromosomes. A comparison of these figures with Figure 7 which is a metaphase chromosome reveals similar patterns of coiling and looping.

These three means of packaging can greatly condense the total DNA length. For example, coiling of DNA exists even in 66 Å chromosome fibers which have been classified as "uncoiled" fibers. If the total length of DNA in 46 anaphase human chromosomes is 180 cm and the volume of these chromosomes is 172.5 u^3 (see INTRODUCTION), the linear length of the DNA within the 66 Å fiber may be calculated by solving for h in the equation, $V = \pi r^2 h$, which is the volume of a cylinder. The length of the DNP molecule, h., is 50.3 cm. Thus the ratio of DNA linear length to DNP linear length is 180/50.3, or 3.6. This indicates that DNA is coiled and packaged in the 66 Å fiber to the extent that the DNA linear length is 3.6 times the DNP linear length. Similarly the DNA:DNP ratio for the 37 Å fiber is 1.1. This means that with extraction of histones by salts the DNA fiber becomes less coiled. When this less obvious form of DNA packaging is combined with visible coiling and folding of chromosome fibers, substantial amounts of DNA condensation occur.

Pairing of 66 Å and 37 Å fibers was observed (Figure 47); and such fibers are interpreted to be single Watson-Crick molecules with greater and lesser amounts of associated protein. The combination of 66 Å fibers is probably very stable because such pairs were not frequently seen in cells dissociated with KCl or with EDTA. This combination is more dependent on proteins than on divalent cations because 66 Å fibers were more numerous in dissociated cells treated with NaCl or citric acid-NaCl than in cells not treated with these agents. The evidence for a primary order of pairing is convincing, but no higher orders of pairing, i.e., multistrandedness, were observed.

Chromosome models

Although these data provide insight into chromosome fibers and their patterns of packaging, no one chromosomal model is implied. Therefore, three models which are compatible with the observations will be discussed. The most important observations in formulating these models were pairing of two 66 Å fibers, coiling, folding, and looping.

The first model is a bineme chromosome. The justification for this model is the pairing of 66 Å and 37 Å fibers which was repeatedly seen. Other electron microscopic observations of paired unit chromosome fibers have been made by Ris (9) and Solari (15). Such a model is compatible with light microscopic, autoradiographic, and irradiation observations of multistrandedness. However a bineme model requires a mechanism to achieve semiconservative replication; linkers which join parallel DNA molecules end to end and which function as a single unit during assortment of mitosis and meiosis would meet this requirement.

The second model is a circular chromosome. Two portions of the circle could associate and thus produce an apparently double 112 Å fiber. Additional reasons for considering such a model are the observation of circular DNA in boar sperm (92) and the presence of circular "chromosomes" in bacteria and viruses. This model could explain the observation of chromatid isolabeling if one portion of the circle was broken and translocated to the other chromatid while the portion with which it was associated remained intact. (However, this would require two breaks to free a given portion of DNA.) If intrafiber association is real, it must have some biologic meaning. Perhaps lateral association of two fibers in firm combination provides more efficient packaging and more effective stability of the chromosome fiber.

The third model is a unineme chromosome. This model would rely entirely on coiling, folding, and looping to attain efficient packaging. The observation of paired fibers could be explained if extensive looping and folding of chromosome fibers was present, e.g., an accordion-like structure. This model is most compatible with genetic and replication requirements.

In summary, present information both in the literature and in this thesis does not permit an unequivocal choice of a chromosome model.

SUMMARY AND CONCLUSIONS

The fibers of both interphase and metaphase chromosomes had a similar appearance. They were long, often extending 5 to 15 μ; and they exhibited varying degrees of coiling. The longitudinal continuity of the fibers was destroyed by DNAase but not by trypsin, chymotrypsin, RNAase, 2.0M NaCl, 0.2N HCl, or 0.1M citric acid-0.125M NaCl. Two fiber populations were defined by measurement of uncoiled fiber diameters: 66±12 Å and 112±18 Å. A third fiber population of 37±6 Å diameter was observed only after exposure of dissociated chromosomes to 2.0M NaCl for 60 minutes or to 0.1M citric acid-0.125M NaCl for 60 minutes followed by 0.2N HCl for 60 minutes. Organizational patterns of chromosome fibers which were observed included pairing of 66 Å and 37 Å fibers, coiling, folding, and looping.

It is concluded that DNA macromolecules form the backbone of chromosome fibers and that such molecules must be intact to maintain longitudinal fiber continuity. It is suggested that the primary unit of human chromosomes is a nucleoprotein fiber of about 66 Å diameter, that such a fiber is composed of a single Watson-Crick DNA molecule with associated protein, and that two such fibers form the 112 Å fiber. Packaging of such chromosome fibers into chromosomes is accomplished by more than one mechanism, but coiling and pairing are probably the two most significant means of such organization.

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TABLE 1

Composition of Nuclei and Chromosomes

Bibliographic Reference	Cell Type	Stage of Mitosis	DNA	RNA	Protein Histone	Protein Non-histone	Protein Total	Lipid	Cation
Mirsky & Ris, 1947 (33)	calf thymus lymphocyte	interphase (isolated "chromosomes")	37%*	1.2%**	55%	8%	63%		
			1 ****	.032	1.5	.2	1.7		
1951 (56)	calf thymus lymphocyte	interphase (isolated "chromosomes")	39%	1%			58%	2%	
			1	.026			1.5	.05	
Vendrely, et al., 1960 (34)	carp erythrocyte		35.6%		48.6%	15.6%	64.2%		
			1		1.4	.4	1.8		
	pike erythrocyte	interphase (isolated nuclei)	33.4%		49.0%	17.7%	66.7%		
			1		1.5	.5	2.0		
	trout erythrocyte		38.2%		47.8%	14.1%	61.9%		
			1		1.3	.4	1.6		
Frenster, et al., 1963 (35)	calf thymus lymphocyte	interphase (isolated chromatin)	31.8%	1.5%			66.7%		
			1	.05			2.1		
Rose & Frenster 1965 (61)	calf thymus lymphocyte	(isolated nuclei)						5.1%***	
		(isolated chromatin)						4%***	
Umana et al. 1964 (36)	rat liver		12.3%		31.2%				
			1		2.6				
	calf liver	interphase (isolated nuclei)	12.9%		28.9%				
			1		2.2				
	calf pancreas		8.4%		15.1%				
			1		1.8				
	calf kidney		5.7%		12.9%				
			1		2.3				
	lamb kidney		8.2%		17.2%				
			1		2.1				

continued on next page

TABLE 1 (continued)

Composition of Nuclei and Chromosomes

Bibliographic Reference	Cell Type	Stage of Mitosis	DNA	RNA	Histone	Non-histone	Total	Lipid	Cation
Umana et al. 1964 (36) (continued)	Walker carcinoma 256 Ascites mouse tumor	"mitotic" (isolated nuclei)	13.3% 1 13.4% 1		11.2% 0.9 14.1% 1.1				
	Calf thymus		20.2% 1		9.6% 0.5				
Cantor & Hearst, 1966 (37)	L2 mouse ascites tumor	metaphase (isolated chromosomes)	13.5% 1	13.5% 1			68.3% 5.1		
Maio & Schildkraut 1966 (38)	Chinese hamster	metaphase (isolated chromosomes)	14.7% 1	11.3% 0.8	44.1% 3.0	29.4% 2.0	73.5% 5		
Naora, et al. 1961 (39)	calf thymus lymphocyte	interphase (isolated nuclei)							Ca 0.024% Mg 0.115%

* per cent dry weight of isolated material
** underlined values have been calculated from original data presented by the authors
*** calculated from data in references 35 and 61
**** non-percentage number is the ratio, X/DNA; e.g., histone/DNA

TABLE 2

Ultrastructure of eukaryote chromosomes: results of thin-section and whole mount studies

Bibliographic reference	Species, cell type, chromosome type	Chromosome fibers: length diameter	Structural roles of chromosome fiber components: DNAase	RNAase	trypsin	chymotrypsin	HCl	NaCl	Organization of chromosome fiber(s)	Model
De Fobertis, 1956 (1)	grasshopper 1° spermatocyte	(S)*								
	early proph.	49 Å**								
	late proph.	70 Å								
	metaphase	100 Å								
Gall, 1956 (3)	Triturus viridescens oocyte, lampbrush	several cm; 500 A; (W)***							long, single fibers	
Ris, 1961 (5)	T. viridescens oocyte, lampbrush	200 Å (W)							random coiling; two 40 Å fibers form the 100 Å fiber, two 100 Å fibers form the 200 - 250 Å fibers, i.e. pairs of pairs	polyneme
	Octopus vulgaris spermatid	100 Å (S)								
		40 Å (S)								
	calf thymus lymphocyte, isolated nucleohistone	100 Å (W)								
		40 Å (W)								
Ris & Chandler 1963 (9)	T. viridescens, nucleated erythrocyte	200-250 Å (W)					20-40 Å (W)			
		100-120 Å (W)								
		40 Å (W)								
Gall, 1963 (7)	T. viridescens nucleated erythrocyte	long; 400-600 Å (W)							single long fibers	unineme
Moses, 1964 (11)	crayfish, spermatid	100 Å (S)								
	thymocyte, isolated nucleus	100 Å (S)								unineme
	rat, sperm	40 Å (S)								

TABLE 2 (continued)

Bibliographic reference	Species, cell type, chromosome type	Chromosome fibers: length diameter	Structural roles of chromosome fiber components: DNAase	RNAase	trypsin	chymotrypsin	HCl	NaCl	Organization of chromosome fiber(s)	Model
Hay & Revel, 1963 (8)	salamander larvae, interphase	50-75 Å (S)							mesh work	
Osgood, et al. 1964 (10)	human lymphocyte, metaphase	500 Å (W)							coiled parallel fibers	multineme
Du Praw, 1965 (12,16)	honeybee embryo, metaphase & interphase	long 230-250 Å (W)	breaks fibers		30-50 Å				helical twisting; folding	unineme, 'folded fiber'
Du Praw, 1966 (17)	luman lymphocyte, interphase & metaphase	long 230-300 Å (W)							twisting	
Wolfe, 1965 (13,14)	T. viridescens & T. granulosa, nucleated erythrocyte	250 Å (W)	breaks fibers					20-30 Å to 250-300 Å	coiling, occ. pairing	
	bovine kidney tissue culture, metaphase	250 Å (W)	breaks fibers	no effect					tightly coiled irreg. mass	
Solari, 1965 (15)	sea urchin, sperm	90 μ long 40 Å (W)	breaks fibers		smaller, smoother fiber		20 Å		coiling, frequent pairing of 40 Å fibers	
Seeger, 1966 (18)	human lymphocyte, interph. & metaphase	5-15 u long; 112 Å, 66 Å, 37 Å; (W)	breaks fibers	no effect	less coiling	less coiling	66 Å	37 Å	coiling, folding, looping, pairing	

* = thin sectioned chromosome preparation (S)
** = dimensions in Å are all fiber diameters (Å)
*** = whole mount chromosome preparation (W)

TABLE 3

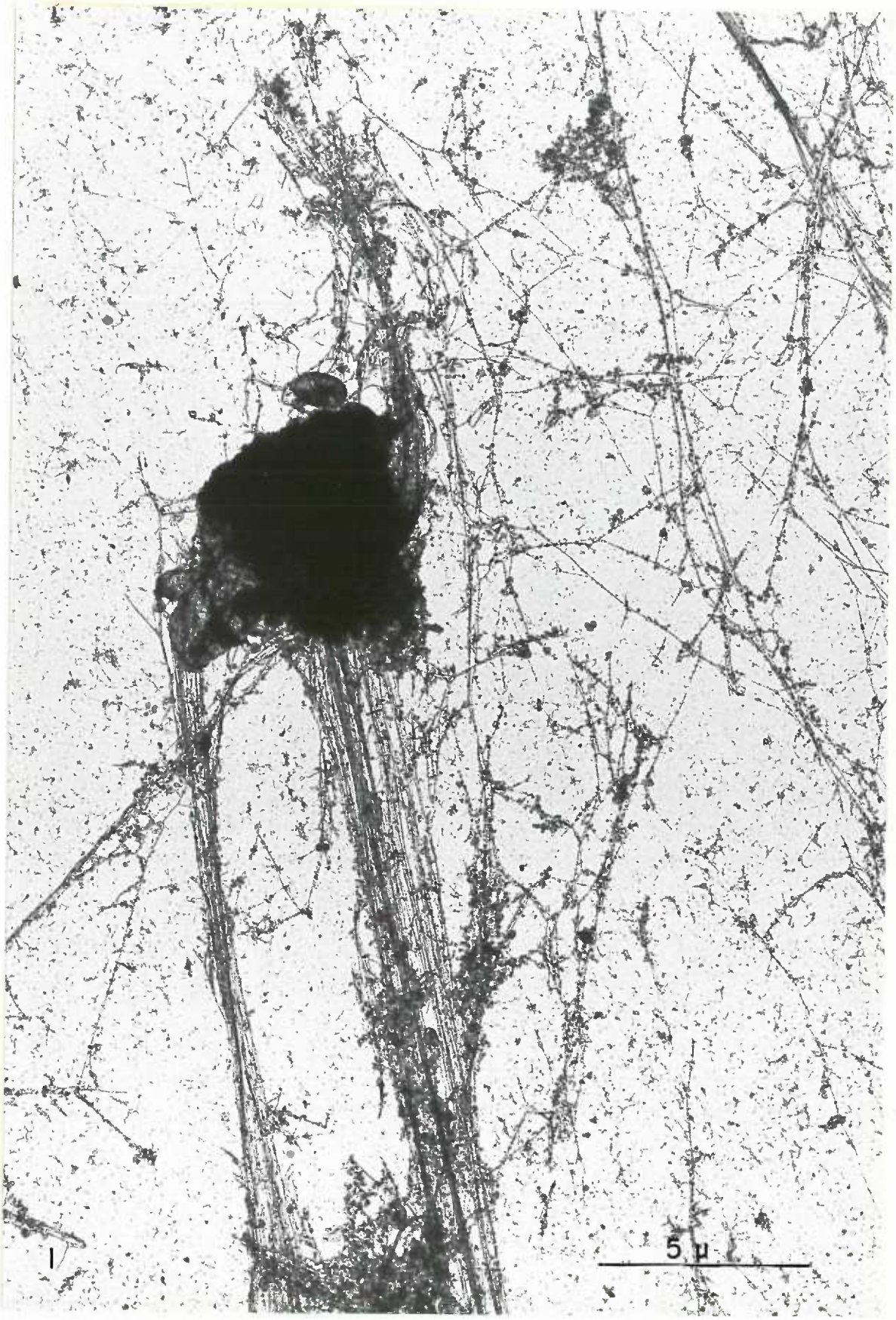
The structural roles of chromosome fiber components and organizational patterns of chromosome fibers. Summary of data. *

Result / Treatment	Spreading and Dissociation	Longitudinal Continuity	Coiling	Fiber Diameter 112 Å	Fiber Diameter 66 Å	Fiber Diameter 37 Å	Association of Fibers in Pairs 66 Å	Association of Fibers in Pairs 37 Å	Folding and Looping of Fibers	Figure No.
KCl	++++	++++	+++	++++	+	0	+	0	+	1,3,7,11,12
K or NaEDTA	+++	++++	++++	++++	±	0	0	0	+++	17-21
Trypsin	++	++++	++++	++++	±	0	±	0	+++	29-33
KCl to DNAase	++++	±	++	++++	0	0	0	0	0	22,23
KCl to RNAase	++++	++++	+++	++++	+	0	+	0	±	24-26
KCl to Trypsin	++++	++++	+	++++	0	0	0	0	0	27,28
KCl to Chymotrypsin	++++	++++	++ (changed)	++++	+	0	+	0	0	34,35
KCl to NaCl	++++	++++	++	+++	++	+	++	+	+	36-42
KCl to HCl	++++	++++	++	+++	++	±	++	±	0	43,44
KCl to Citric Acid-NaCl to HCl	++++	++++	+	+++	++	++		+	0	45,46

* Grading scale: Chromosome fibers which were dissociated and spread in 0.01M KCl are used as the standard for comparison

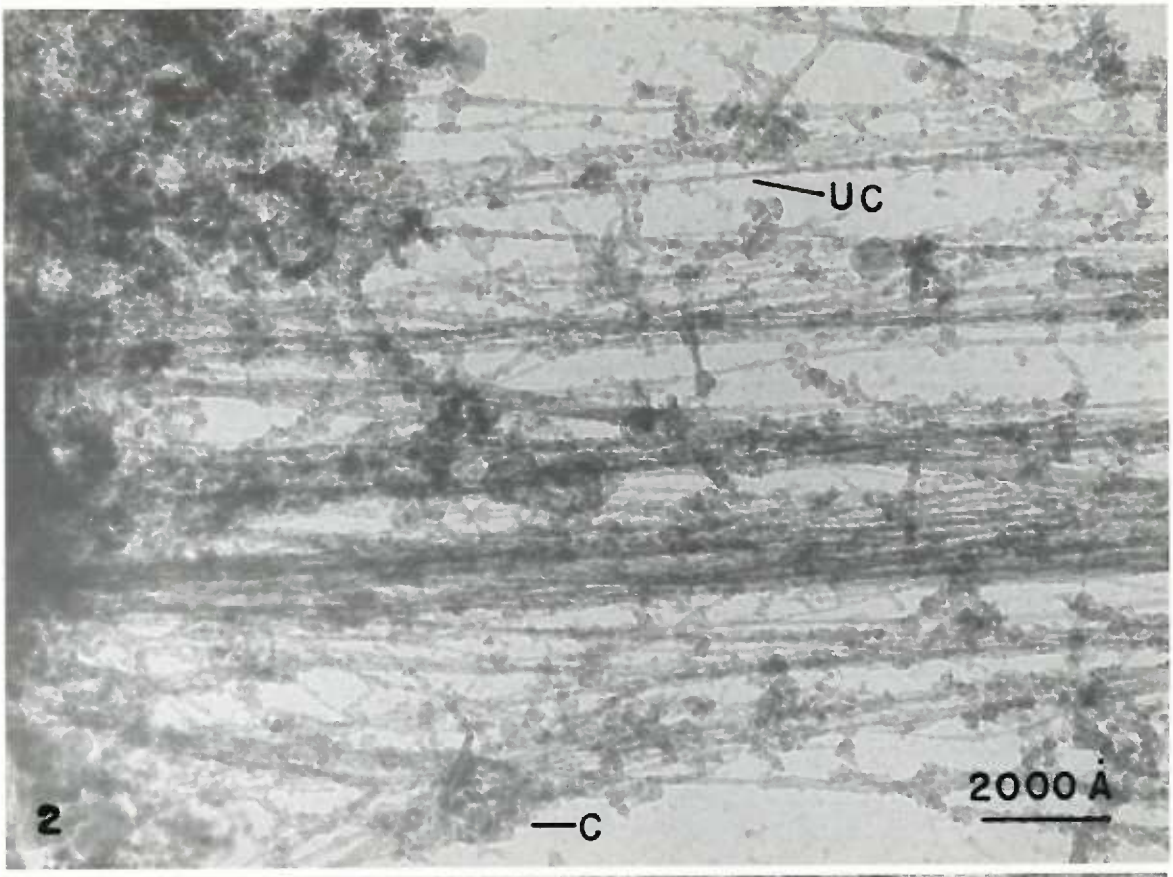
++++ = observed always
+++ = observed most of the time
++ = observed usually
+ = observed occasionally
± = observed equivocally
0 = not observed

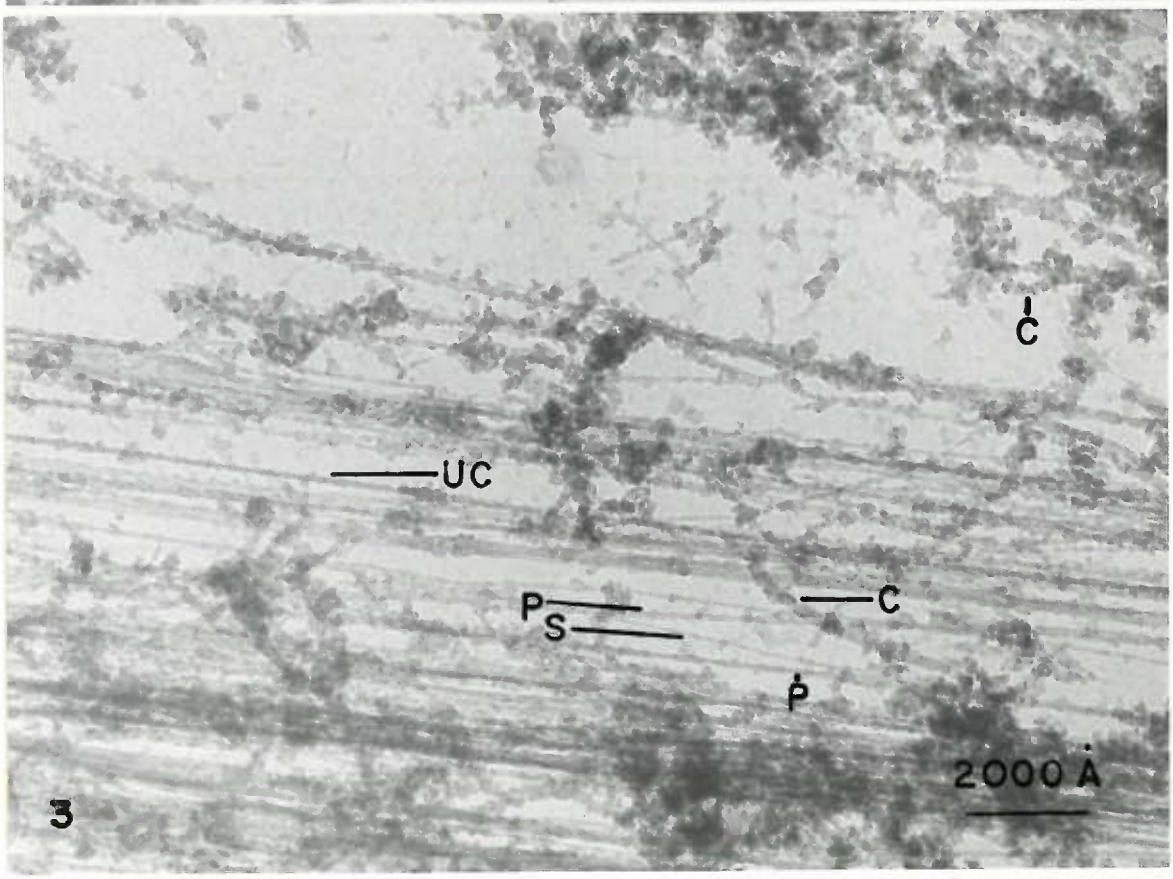
(Figure 1). The chromosome fibers of this metaphase cell have been spread and dissociated in 0.01M KCl. Many fibers which are longer than 5 μ are present (x7,000).


I
5 µ

(Figure 2). At higher magnifications the chromosome fibers (Figure 1) exhibit varying degrees of coiling. Some fibers are highly coiled (C) whereas others are uncoiled (UC) (x79,700).

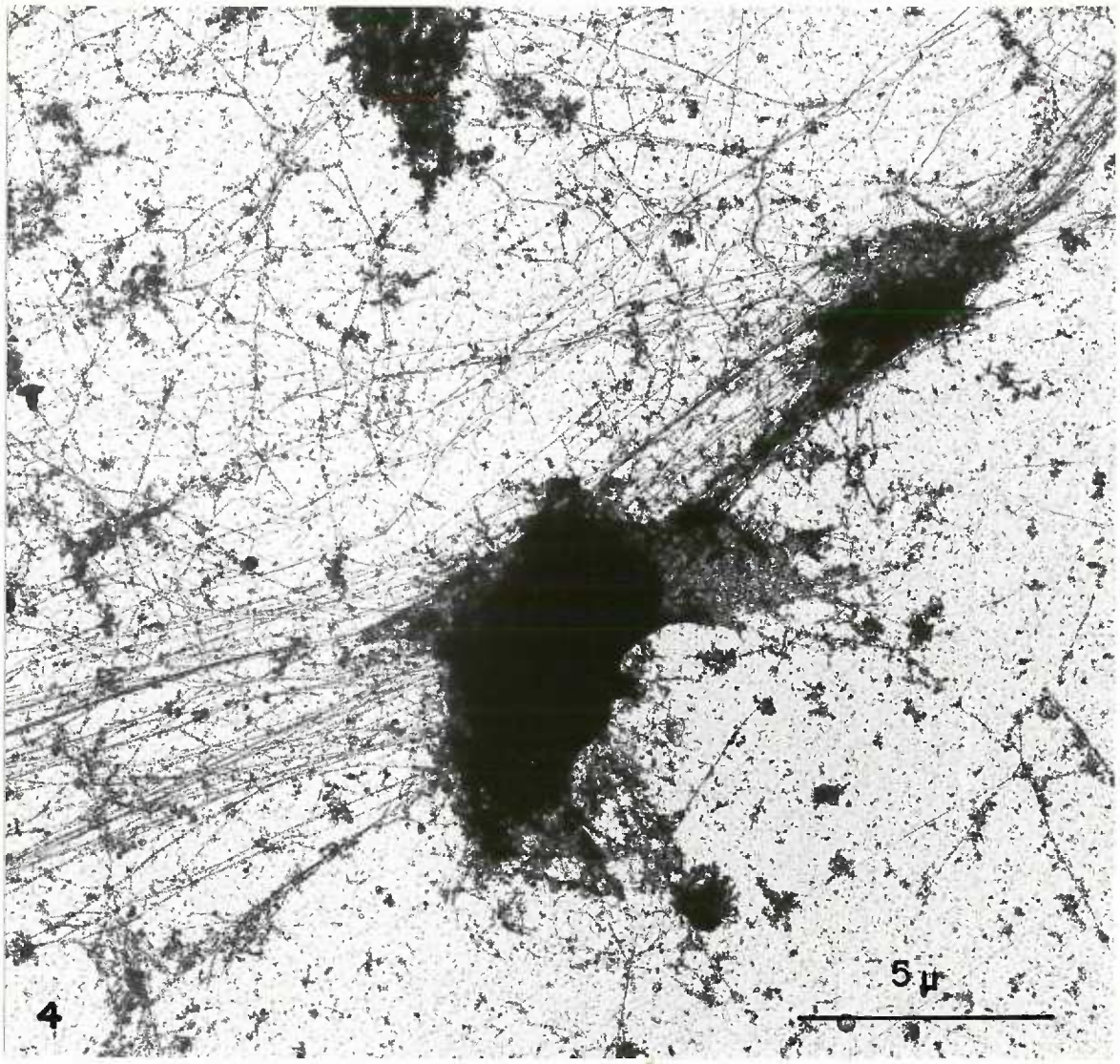
(Figure 3). This portion of the cell (Figure 1) shows coiled (C), uncoiled (UC), 66 Å (S), and 112 Å (P) chromosome fibers (x79,700).

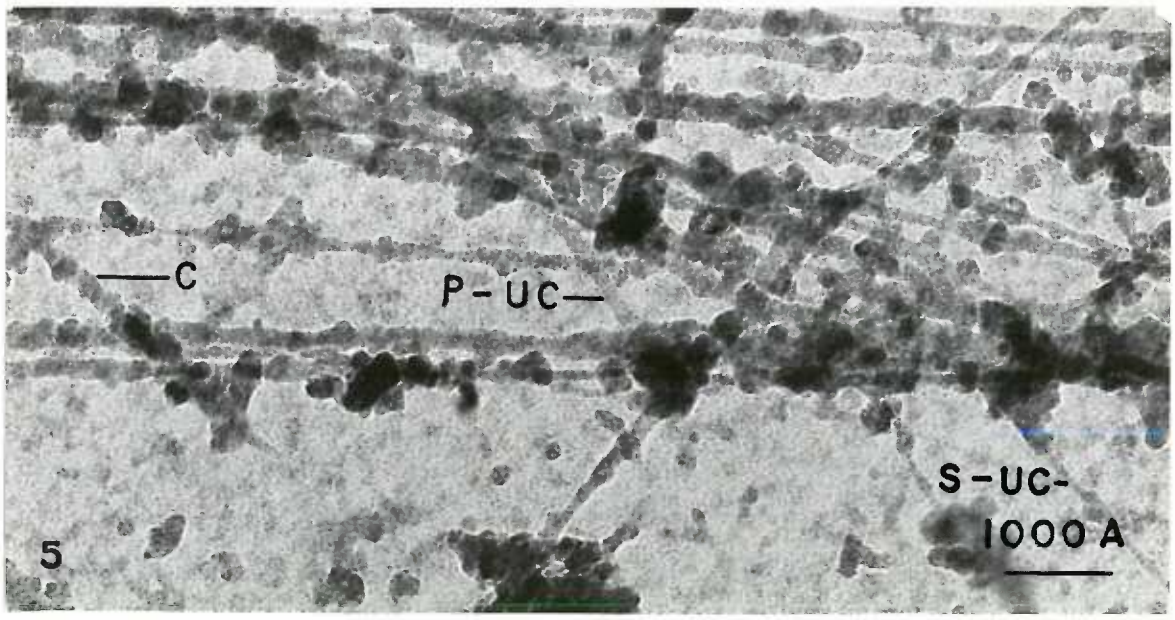

UC
2000 Å
2
C


C
UC
P
S
C
P
2000 Å
3

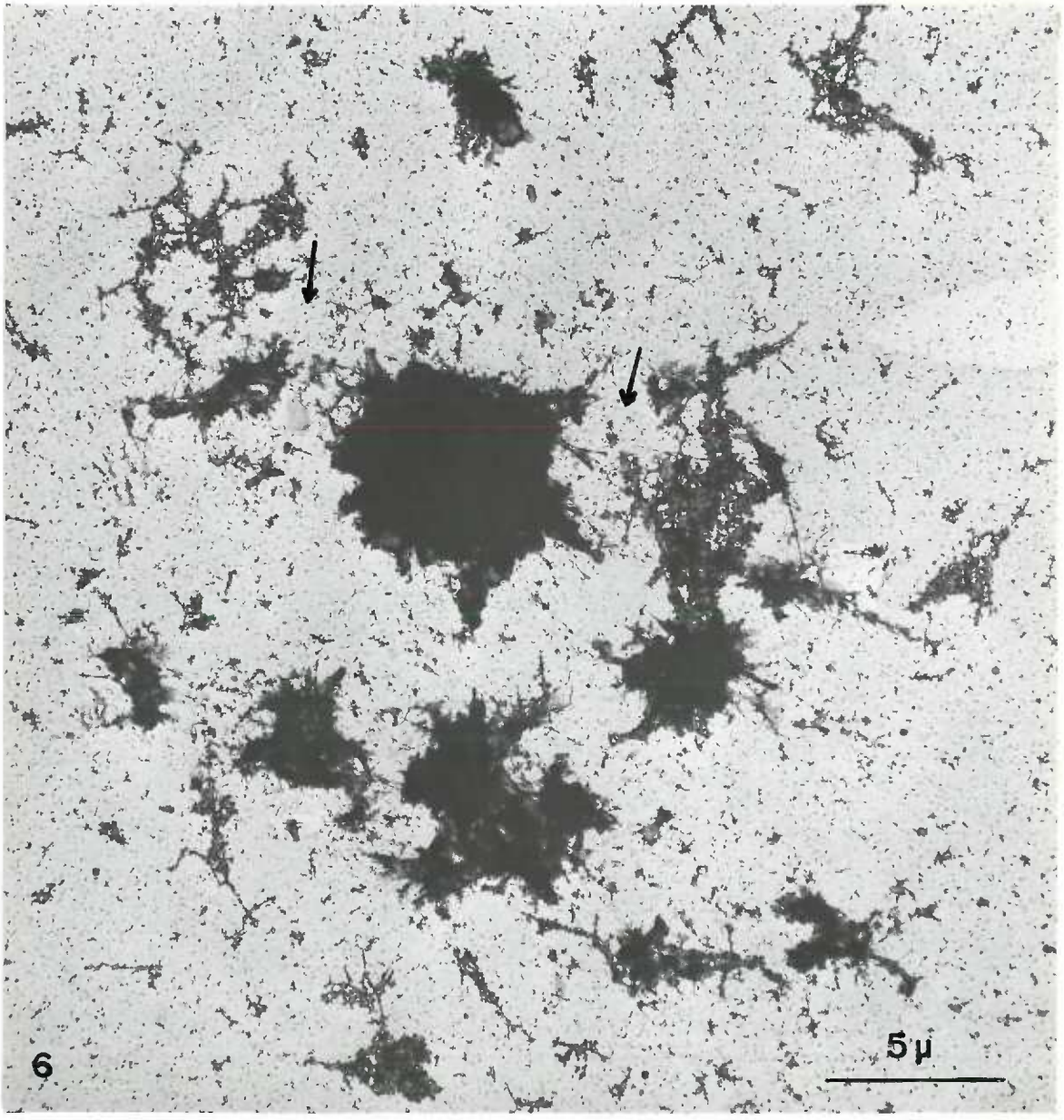
(Figure 4). This is an interphase lymphocyte in which chromosome fibers were spread and dissociated with 0.01M KCl. Many fibers can be followed for lengths of 5 to 10 μ (x7,100).

(Figure 5). At a high magnification (Figure 4) coiled (C), uncoiled (UC), 66 Å (S) and 112 Å (P) fibers are illustrated (x142,000).

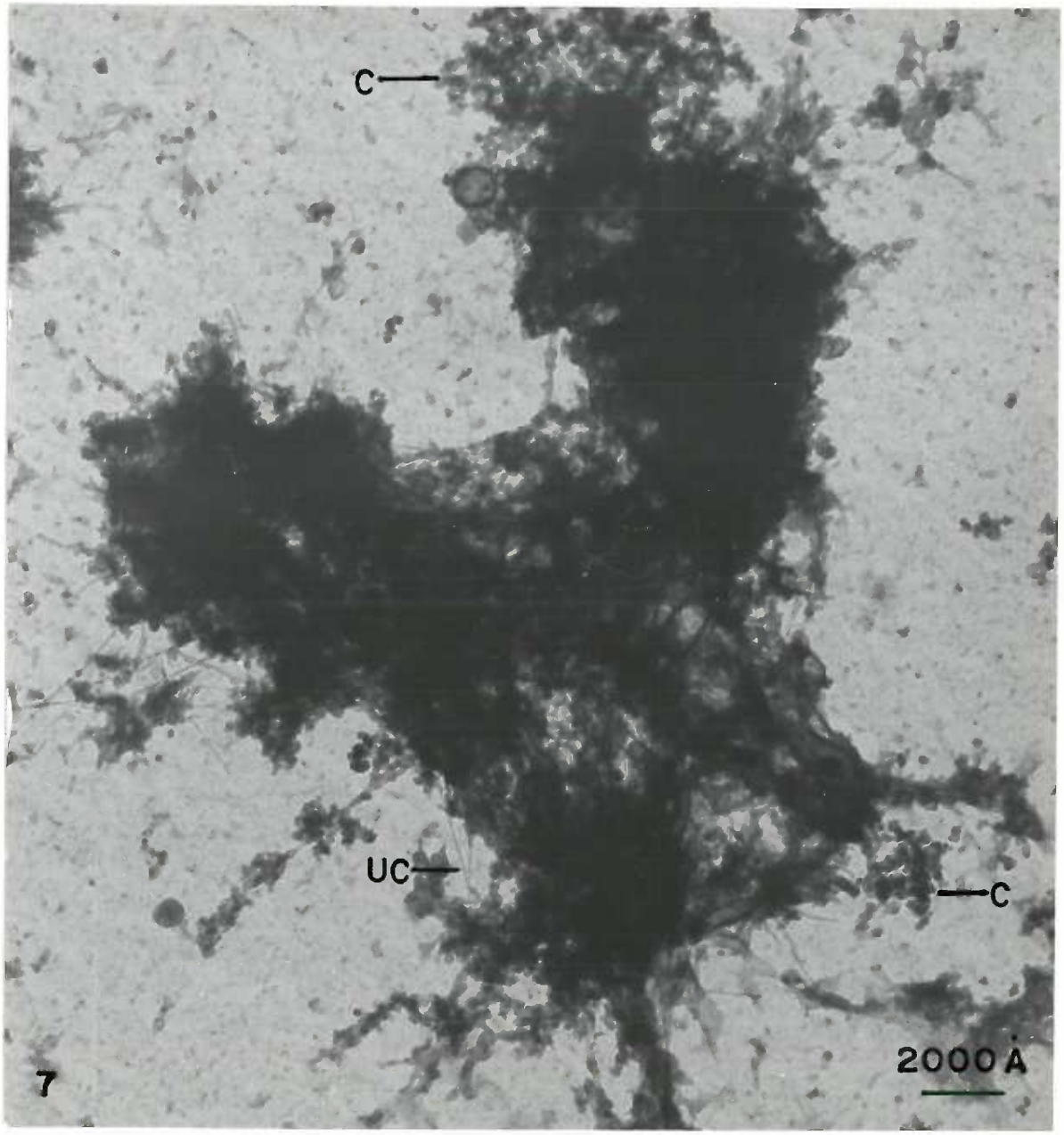

5μ
4


C
P-UC
S-UC-
1000 A
5

(Figure 6). This is a metaphase cell in which chromosomes are only partially spread and dissociated; however, some long fibers are present (arrow) (x4,900).

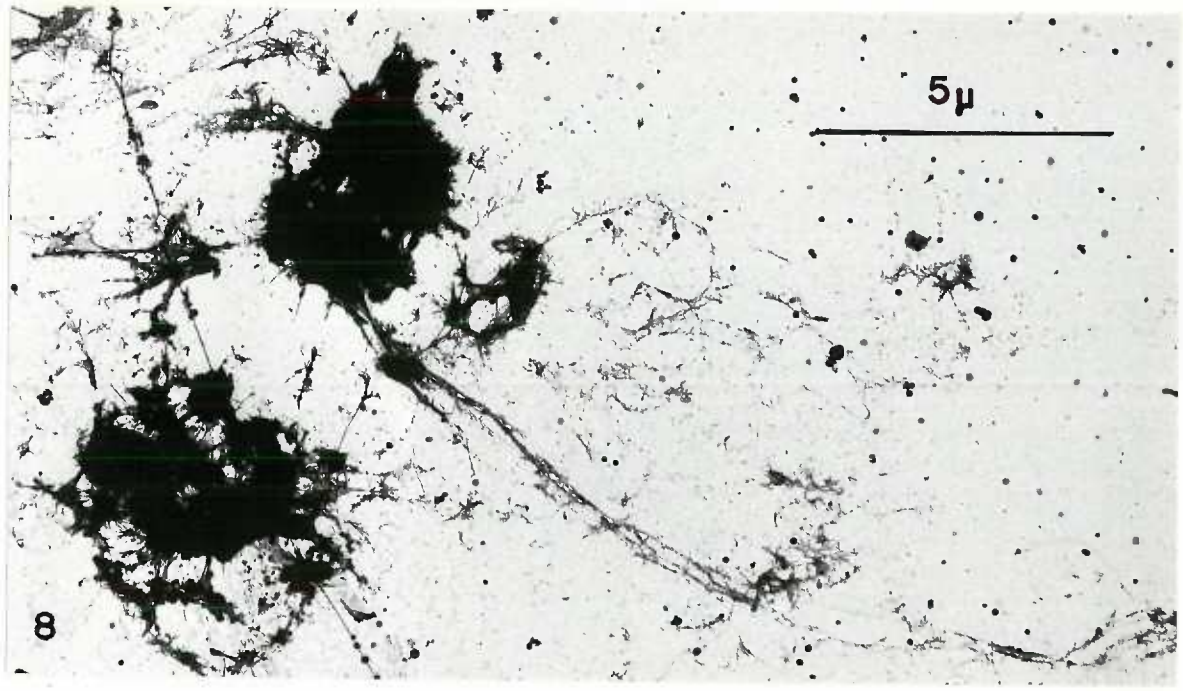

6
5μ

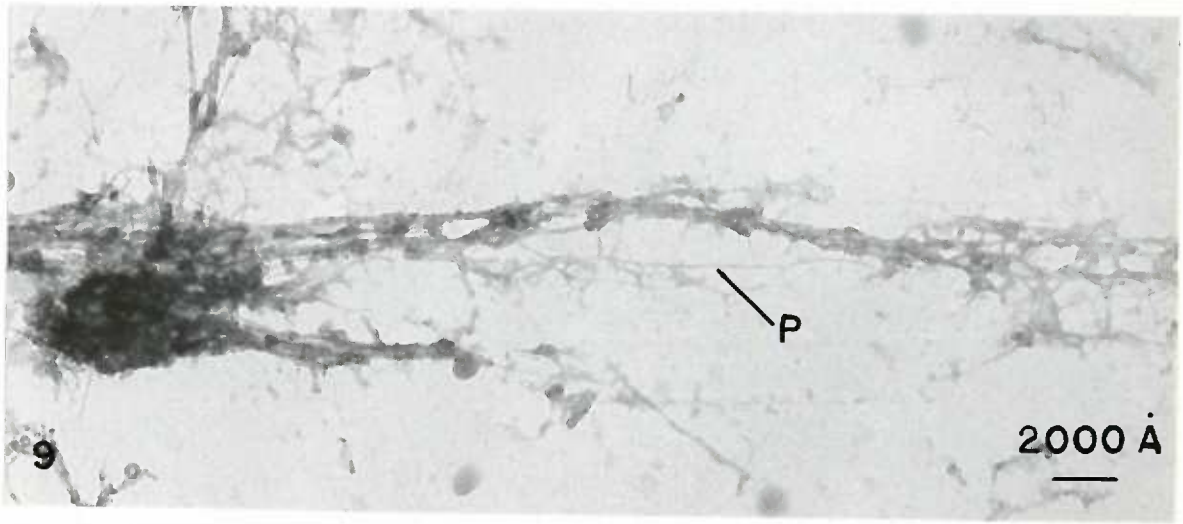
(Figure 7). This metaphase chromosome (from Figure 6) has fibers which are coiled (C) and uncoiled (UC). There is a suggestion that a continuous fiber is present in the coiled areas of the upper right chromatid (x56,950).

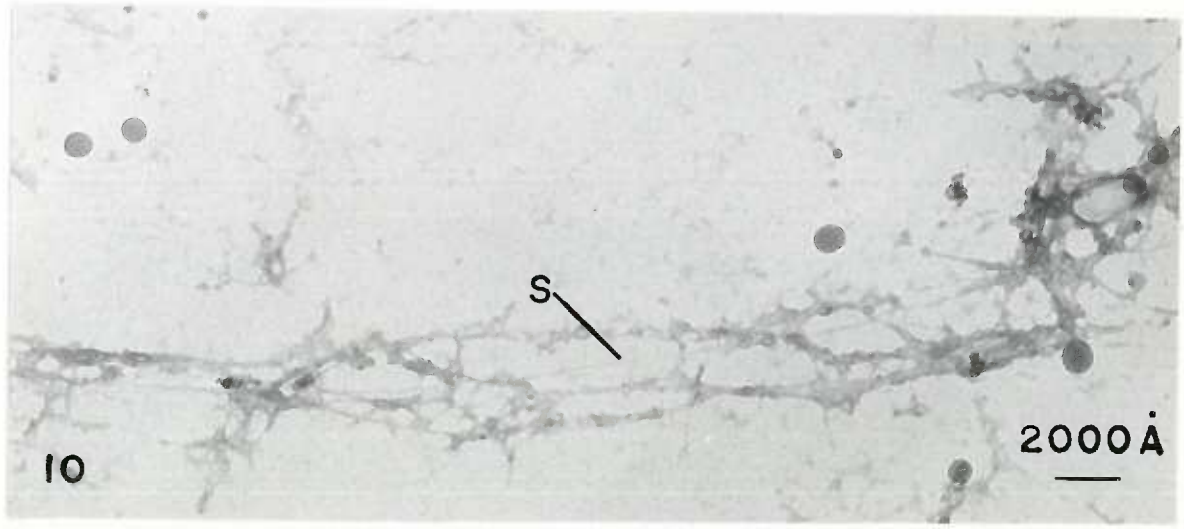

C
UC
C
2000 Å
7

(Figure 8). The metaphase chromosome in this micrograph was dissociated with KCl and subsequently exposed to 2.0M NaCl for $2\frac{1}{2}$ hours. A few fibers extend 10 to 15 micra, running in parallel. (x7,600)

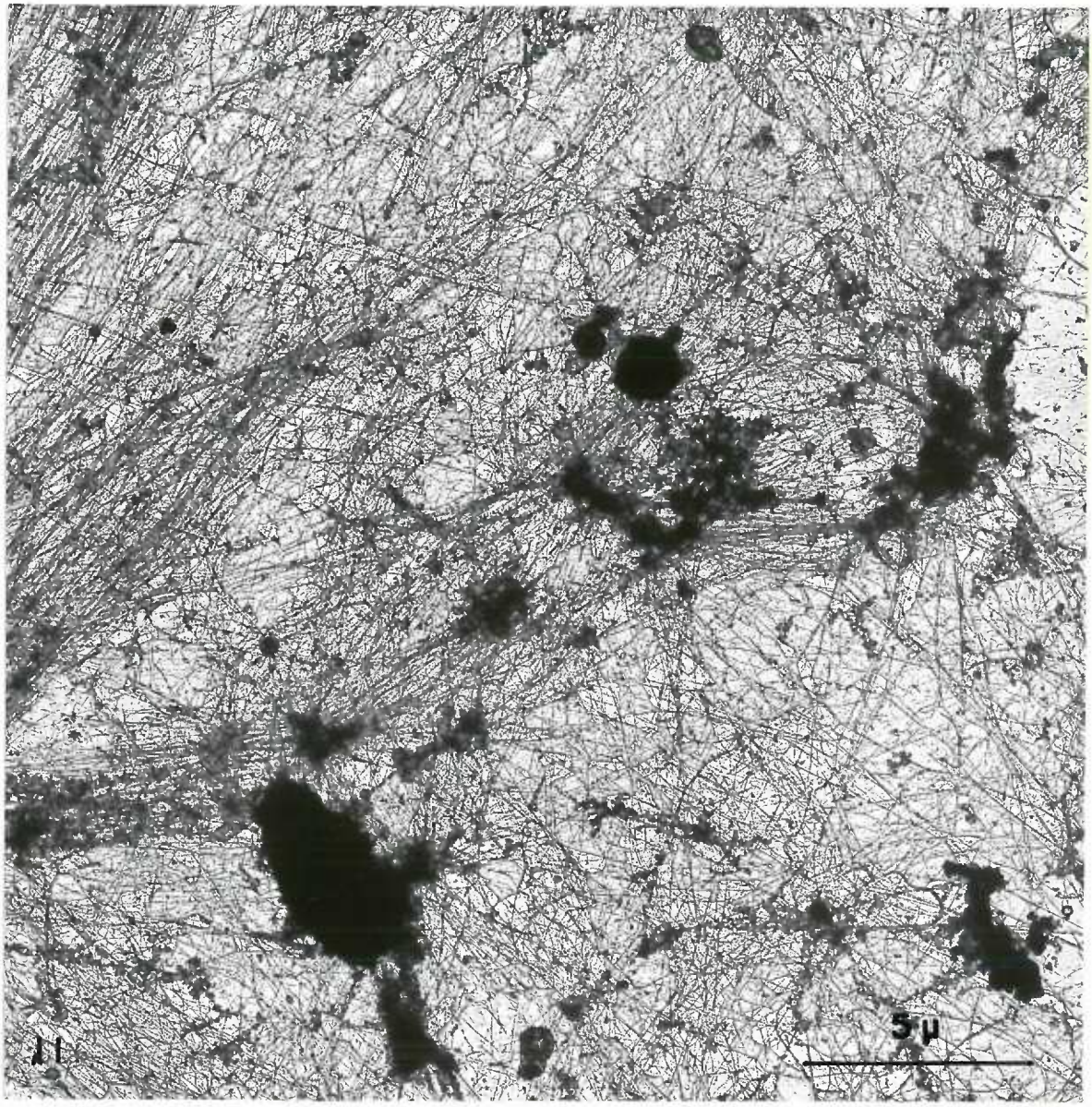
(Figures 9 & 10). Higher magnification of the fibers which extend from the chromosome (Figure 8). The dissociated chromosome fibers (Figure 8) appear to be parallel. There is a large variation in size but 112 Å (Figure 9, P) and 66 Å (Figure 10, S) are seen, (x43,480).


5μ
8

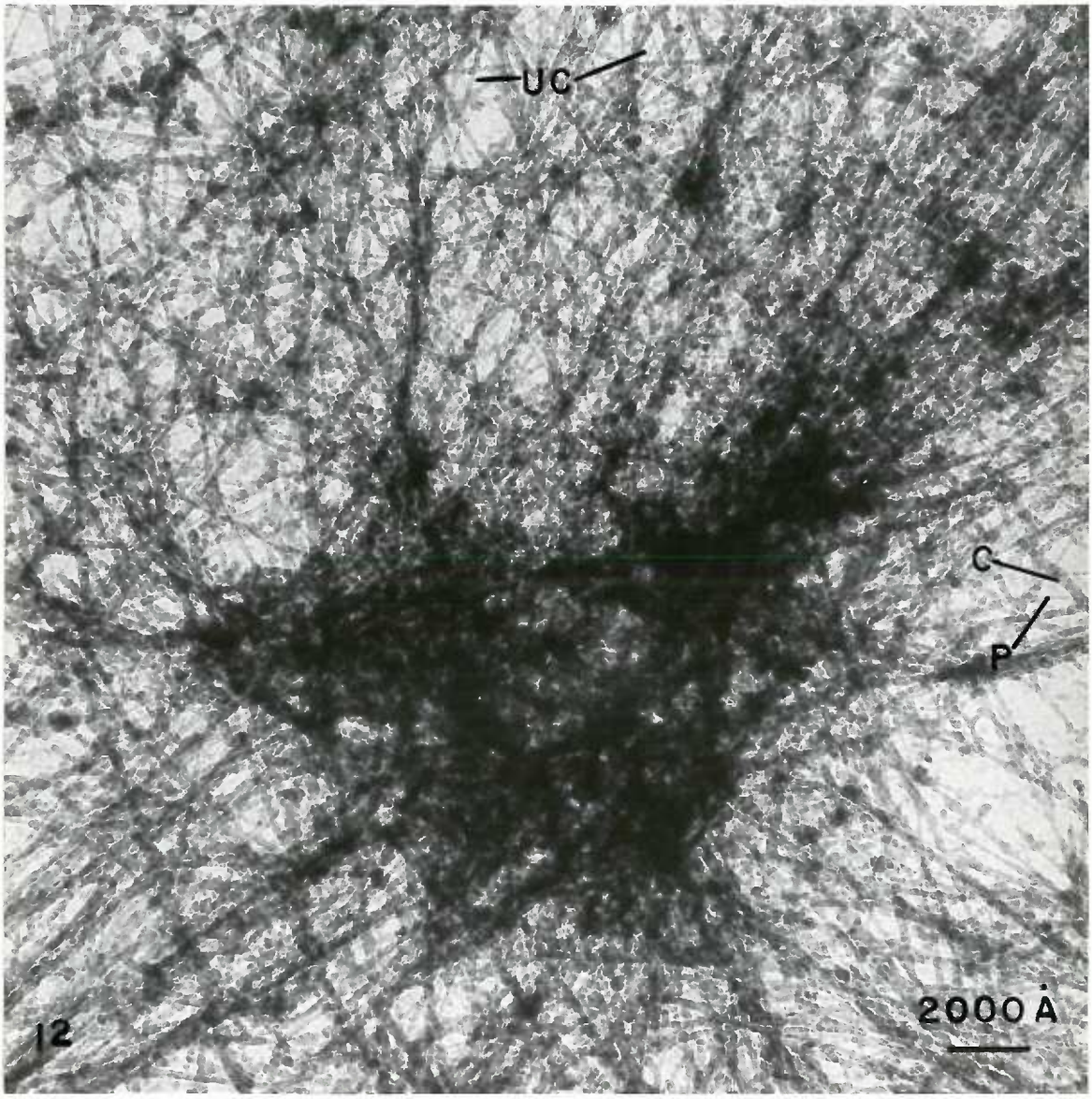

P
2000 Å
9


S
2000 Å
10

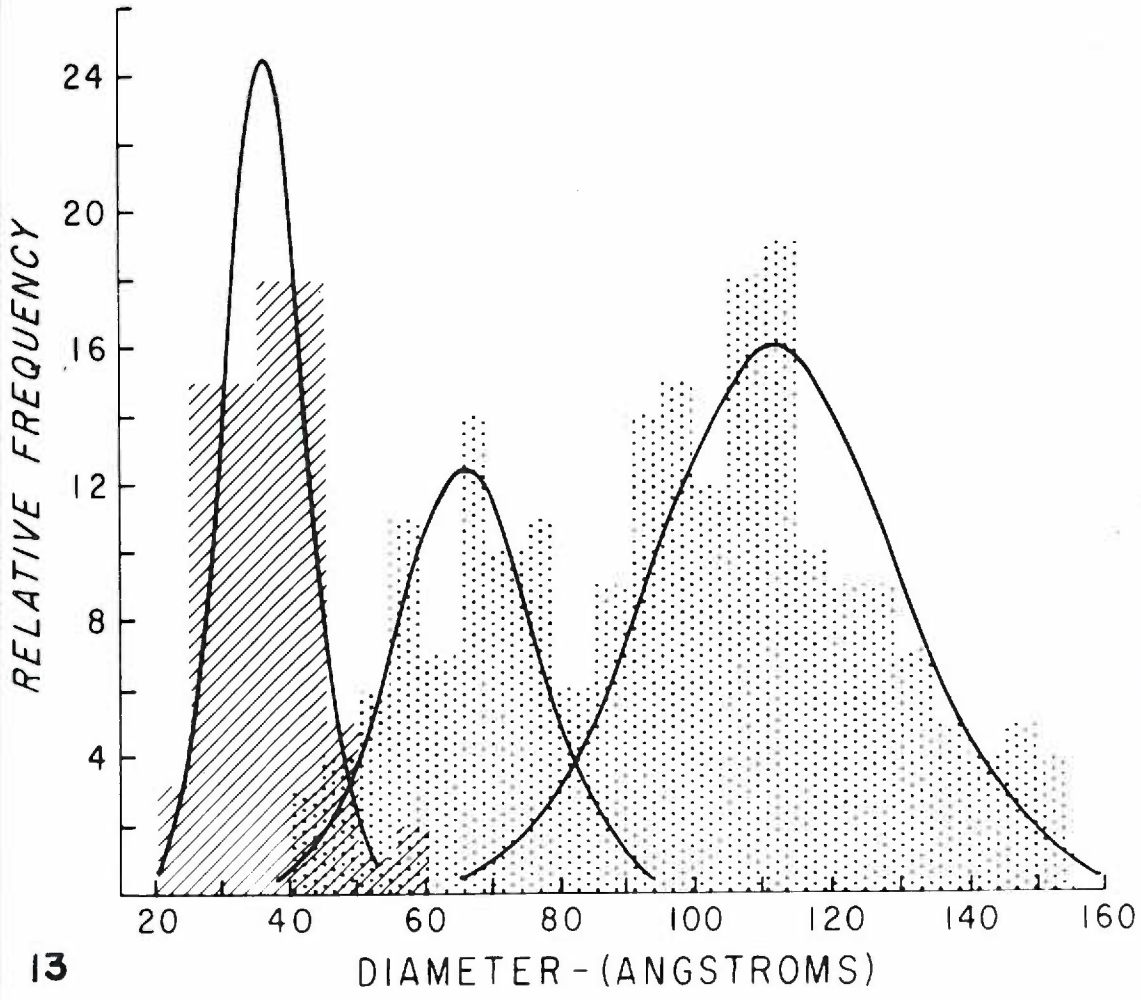
(Figure 11). The chromosomes of this cell, probably metaphase, have been dissociated almost completely by 0.01M KCl. Many fibers which are longer than 5 μ are present (x6,270).


5 μ

(Figure 12). The long fibers shown in Figure 11 are seen to originate from aggregates of fibers. Coiled (C), uncoiled (UC), and paired (P) 66 Å fibers are seen (x53,000).

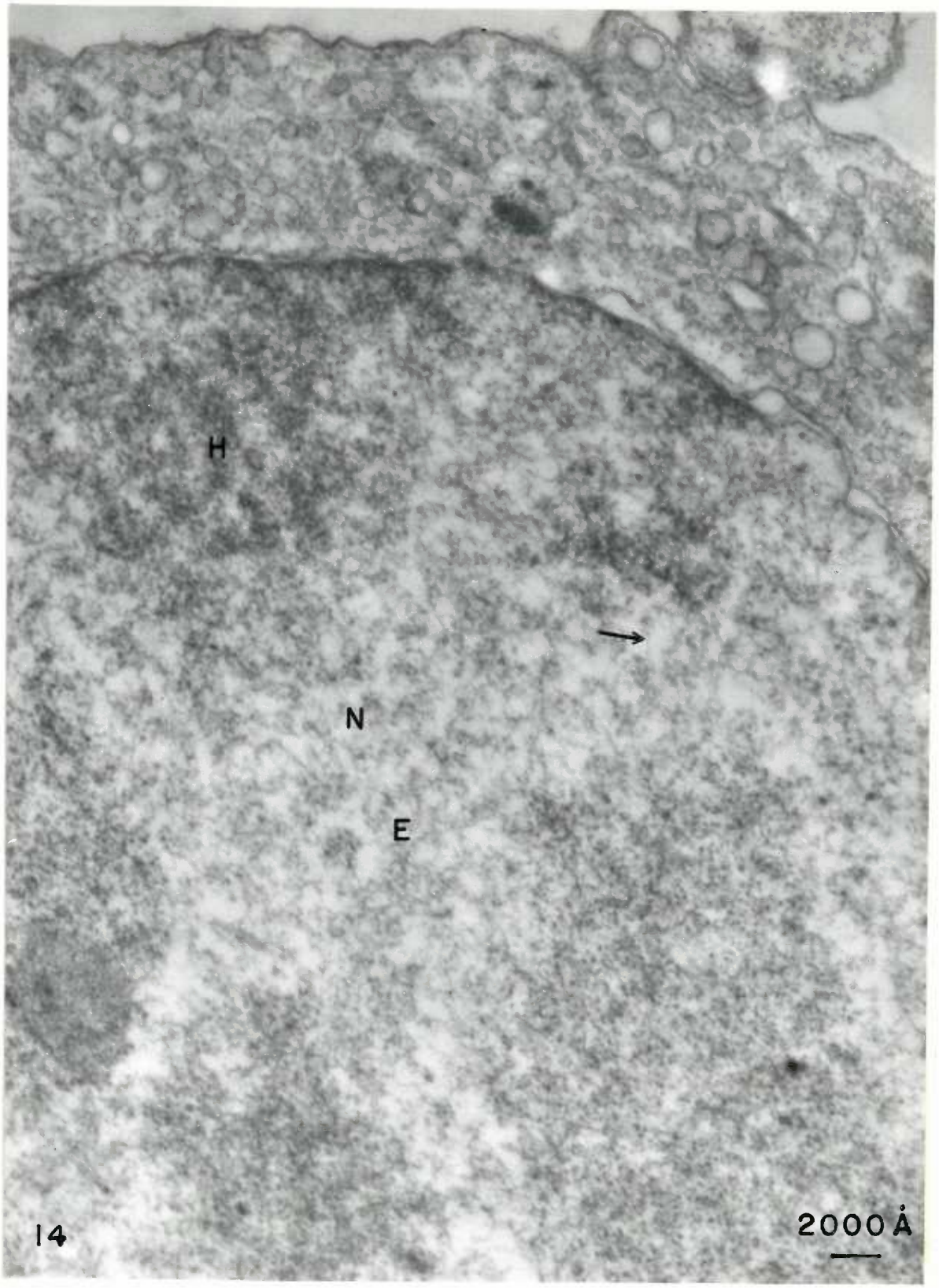

UC
C
P
2000 Å
12

(Figure 13). The relative frequency of diameters of single uncoiled chromosome fibers is illustrated by this histogram. Fibers of the 66 A and 112 Å population (stippling) are seen following exposure of cells to 0.01M KCl, but fibers in 37 Å population (diagonal lines) are seen only after exposure of dissociated chromosomes to 2.0M NaCl or 0.1M citric acid-0.125M NaCl followed by 0.2N HCl. The distributions of the three fiber populations are compared to the distributions of three normal curves which are calculated from the observed values of 37±6 Å and the fitted values of 66±12 Å and 112±18 Å (mean ± s.d.).


RELATIVE FREQUENCY
24
20
16
12
8
4
20
40
60
80
100
120
140
160
DIAMETER - (ANGSTROMS)

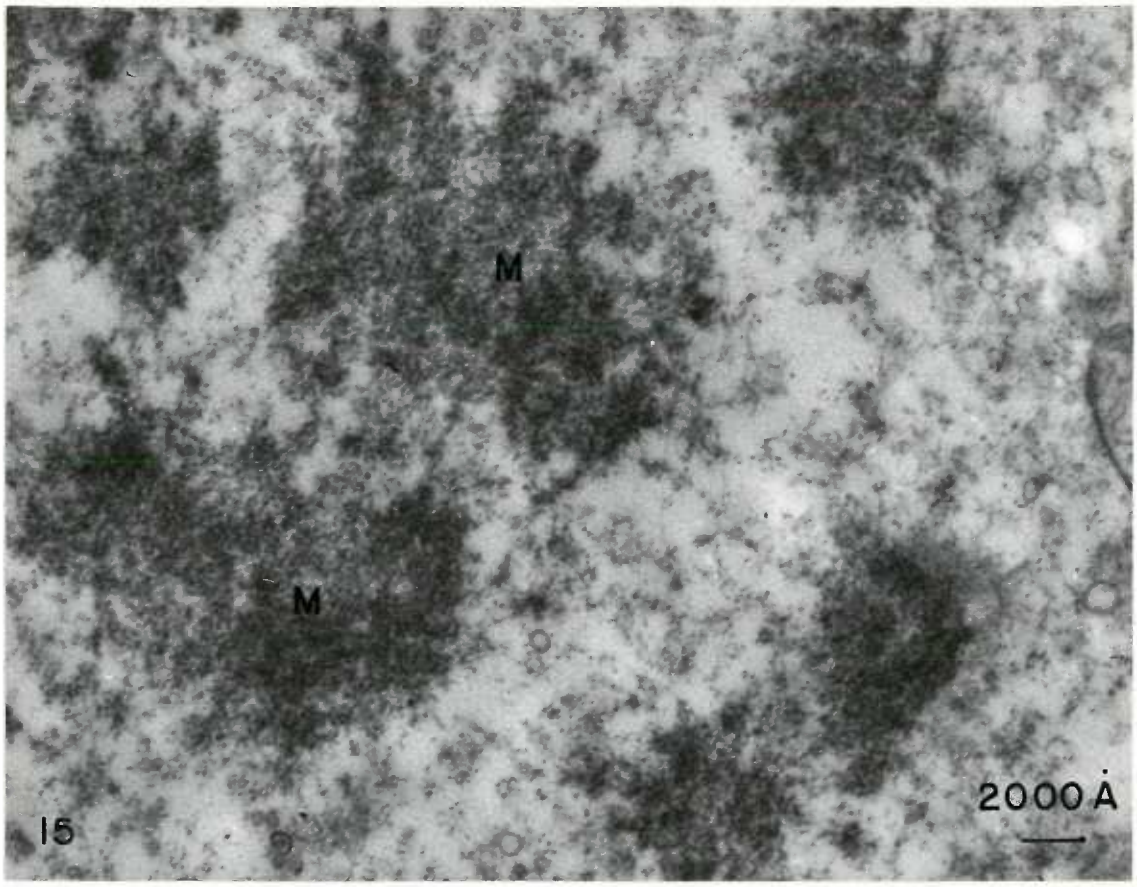
13

(Figure 14). This thin section of an interphase nucleus (N) shows areas of densely packed (H, heterochromatin) and of dispersed (E, euchromatin) chromosome fibers. Granules and short fibers of about 112 Å diameter are seen (arrow); these are sectioned chromosome fibers (x39,800).

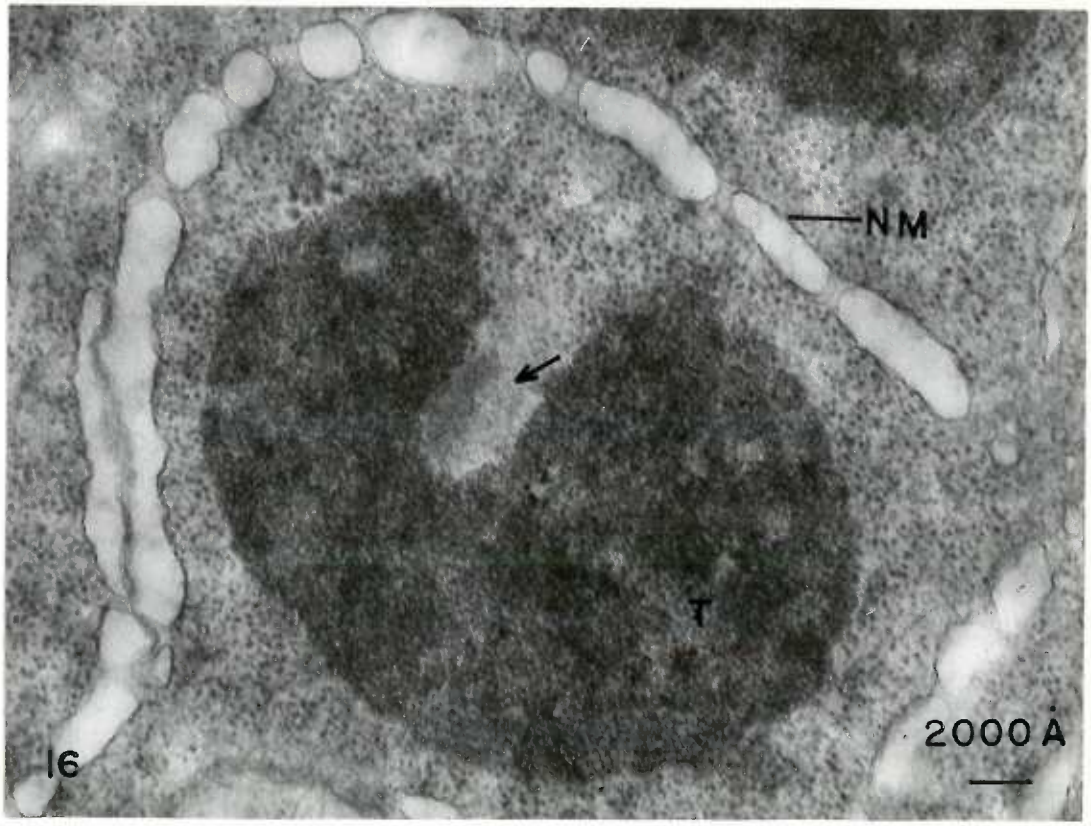

H
N
E
14
2000 Å

(Figure 15). This is a thin section of a metaphase chromosome (M). The chromosome fibers are tightly packaged, and little more than granules is seen (x39,800).

(Figure 16). This section has passed through a telophase chromosome (T) in which some fibers of about 112 A diameter are seen (arrow). The nuclear membrane (NM) is just beginning to reform (x39,800).



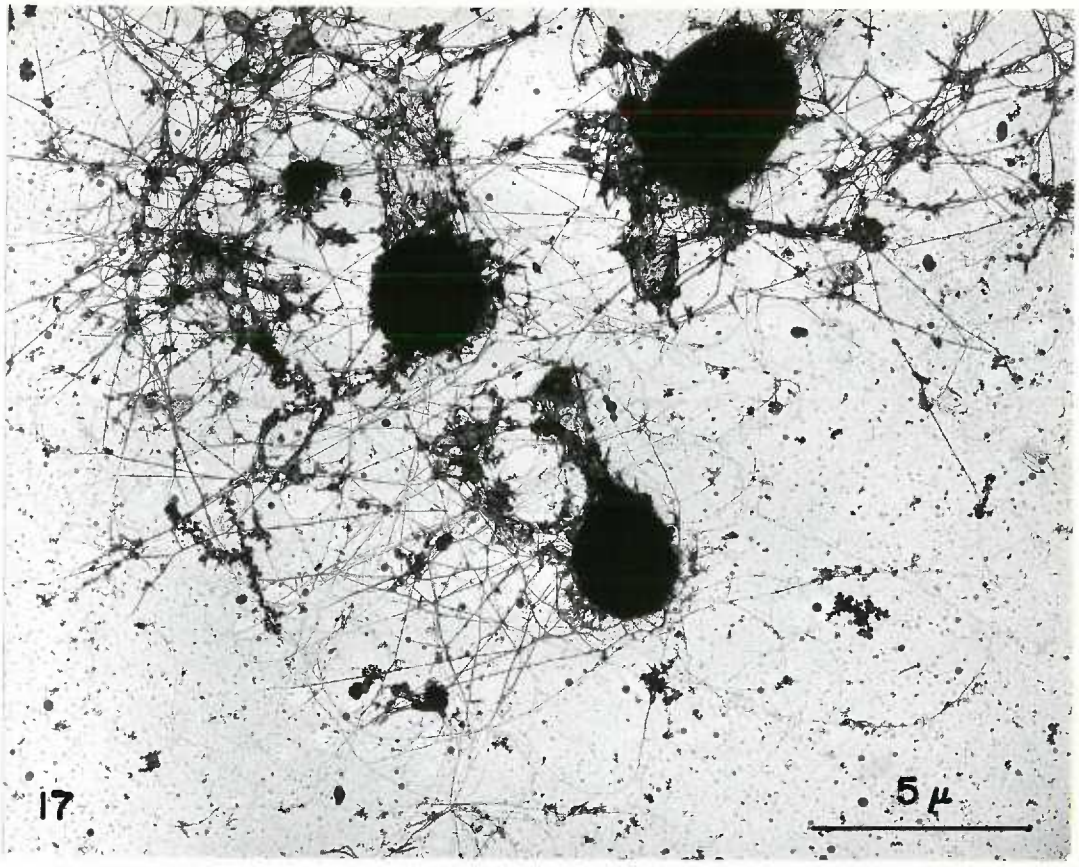
15

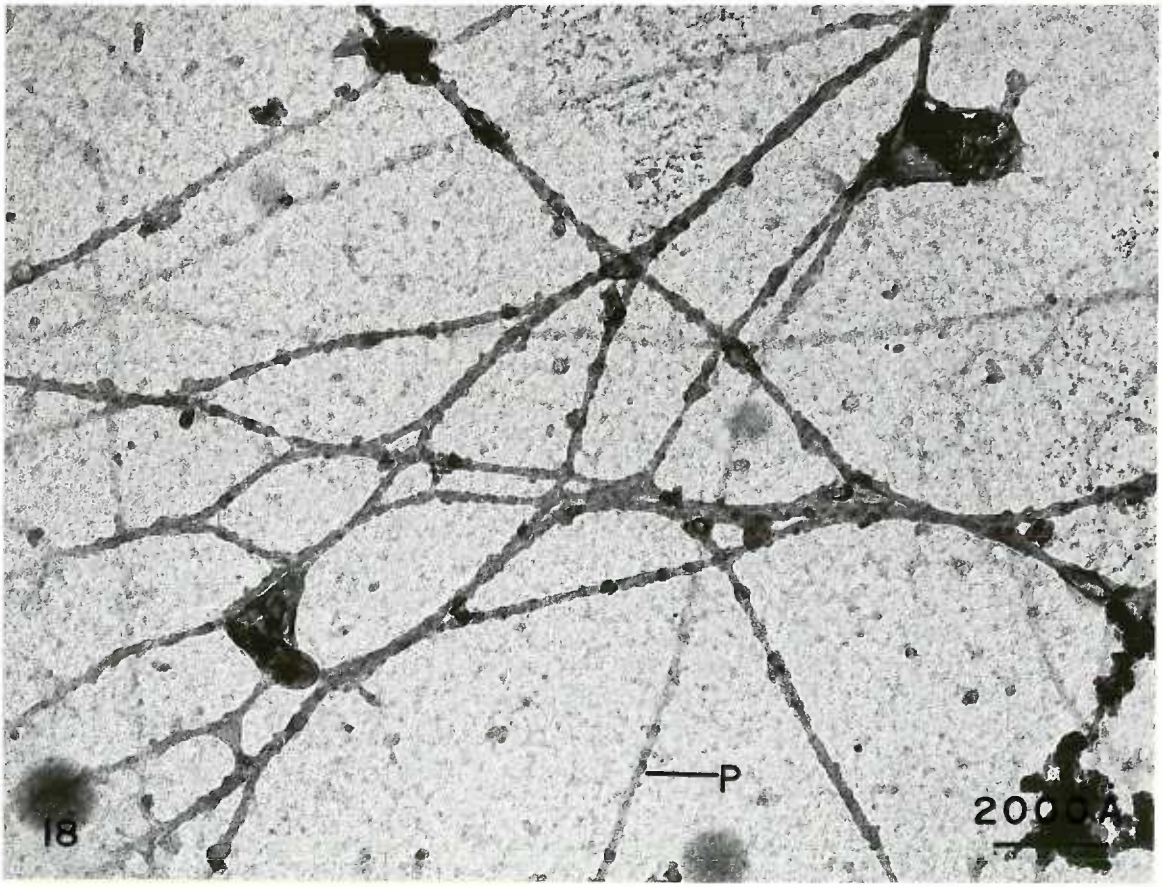


16

(Figure 17). The chromosome fibers of these interphase cells were dissociated in 0.1M EDTA. The dissociation and spreading of the chromosomes is not as marked with EDTA as it is with KCl. Numerous long fibers are present (x5,700).

(Figure 18). At a higher magnification the chromosome fibers of Figure 17 exhibit minimal coiling and have diameters of about 112 Å (P). No 66 Å fibers are seen (x72,600).

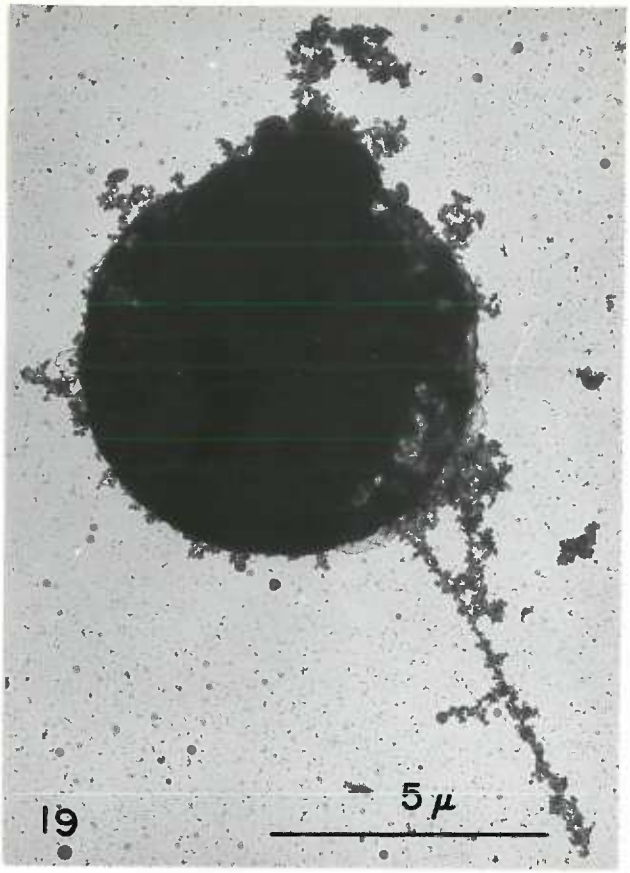

17
5 μ

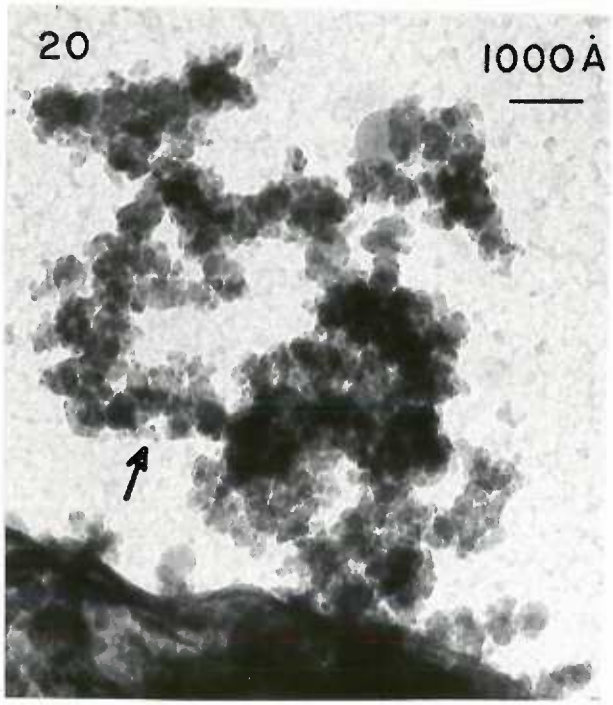

P
18
2000 A

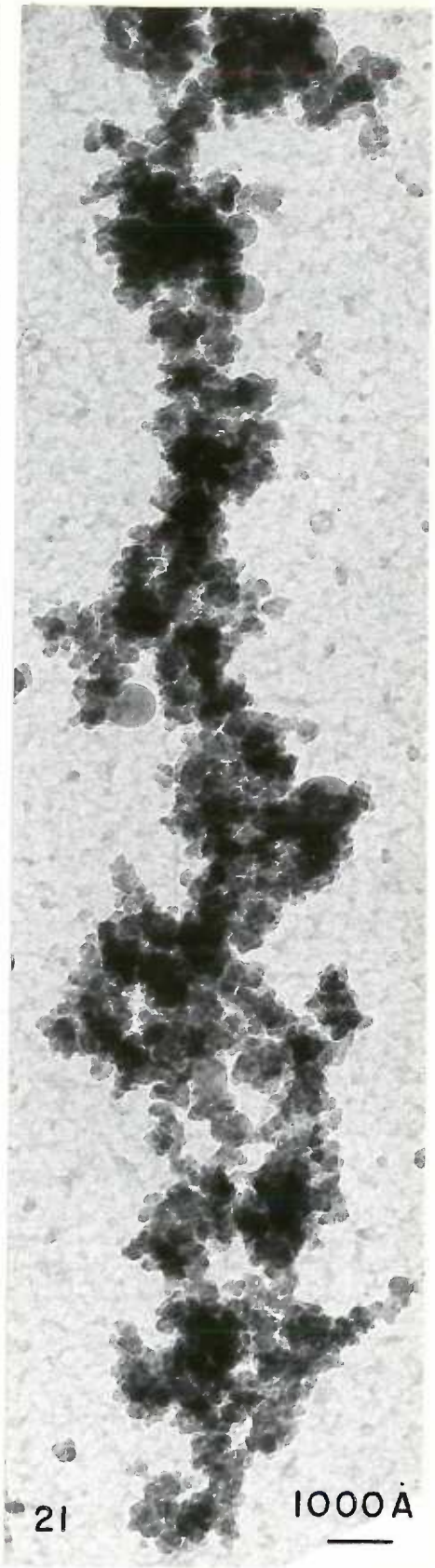
(Figure 19). The chromosome fibers of this interphase cell which was treated with EDTA exhibit minimal degrees of dissociation (x7,300).

(Figure 20). High magnification of the chromosome fiber(s) (Figure 19) is suggestive of a long continuous fiber which is highly coiled and which is folded and looped. At one point a fiber of about 112 Å diameter is seen (arrow) (x92,340).

(Figure 21). This micrograph of chromosomal material illustrates primary coiling with superimposed looping and folding. The overall spiral configuration is suggestive of a secondary level of coiling (x92,340).

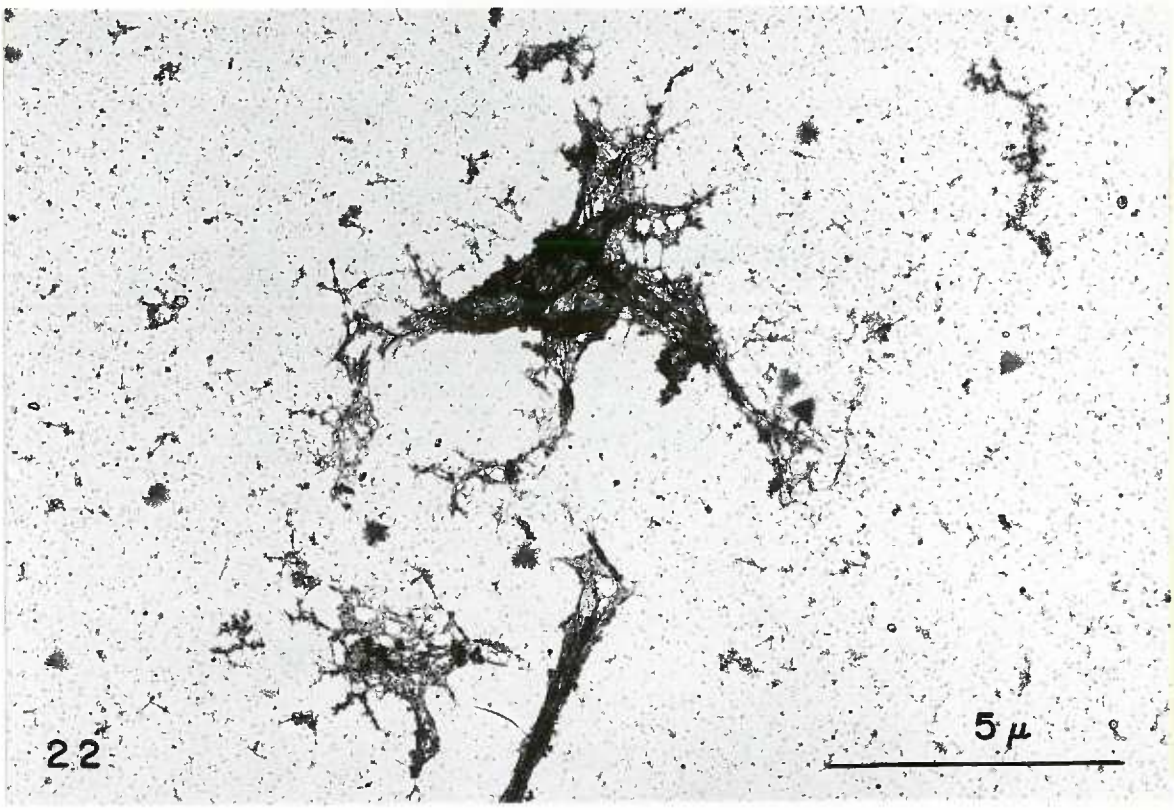

19
5 μ

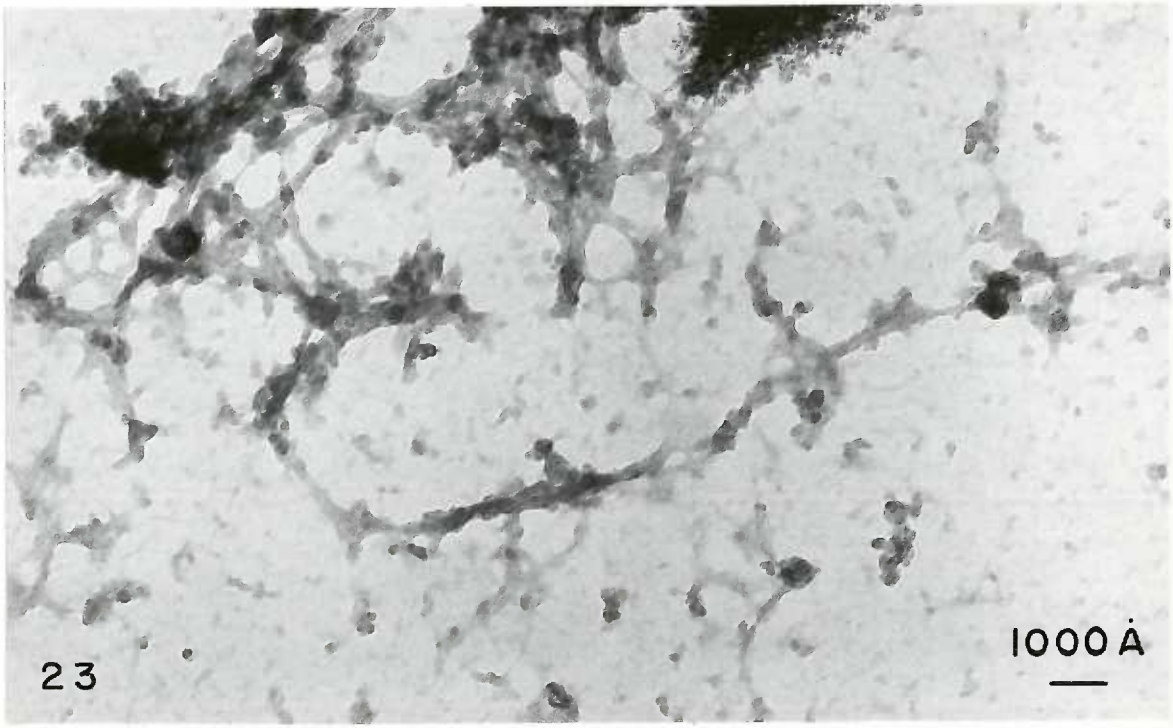

20
1000 Å


21
1000 Å

(Figure 22). This cell, after being dissociated in KCl, was exposed to DNAase. There is a striking absence of long uncoiled chromosome fibers, but highly coiled and aggregated fibers remain (x7,600).

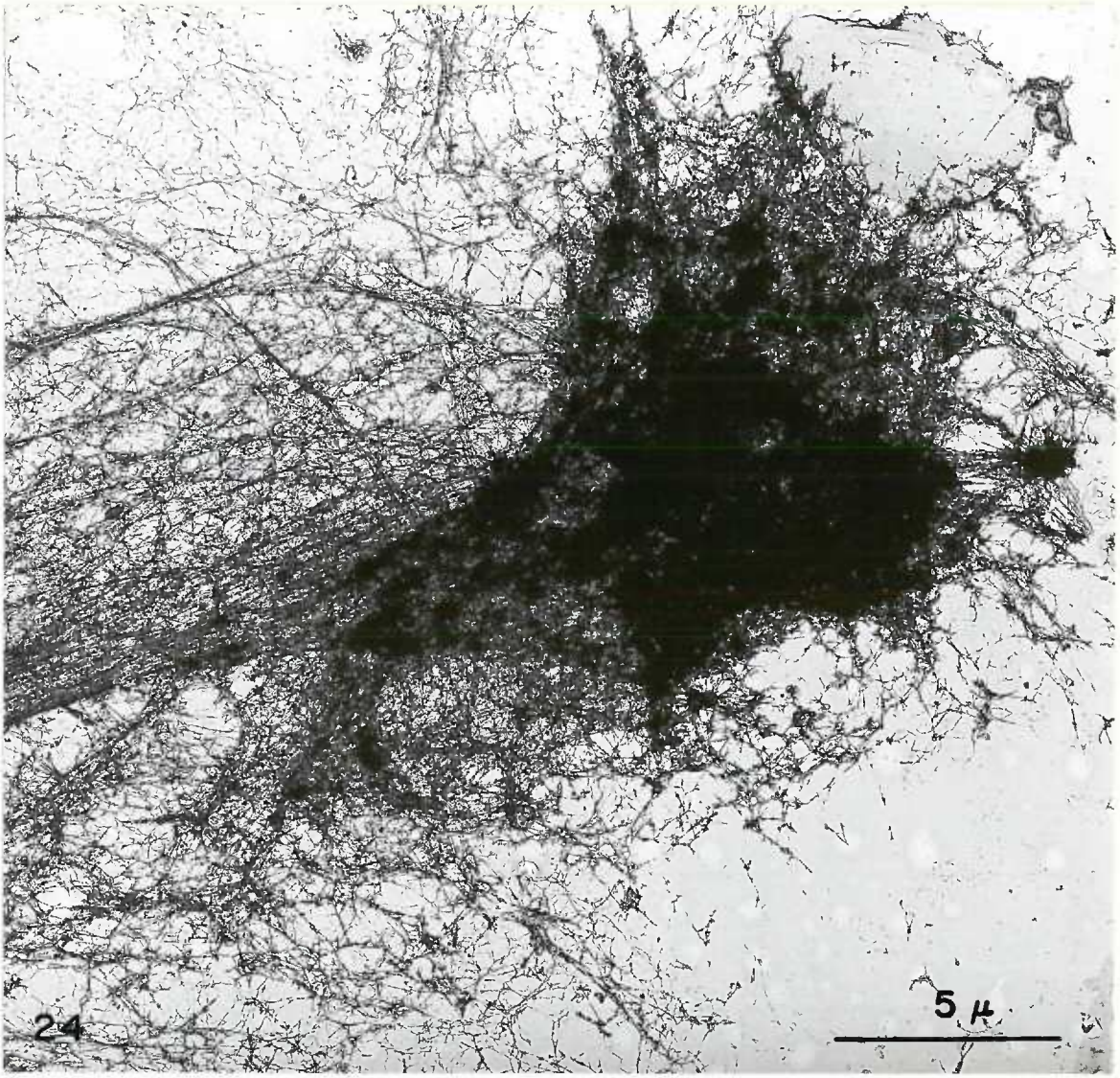
(Figure 23). The disruption of chromosome fibers is demonstrated in this micrograph (Figure 22). Only short segments and aggregates of fibers are present (x72,750).

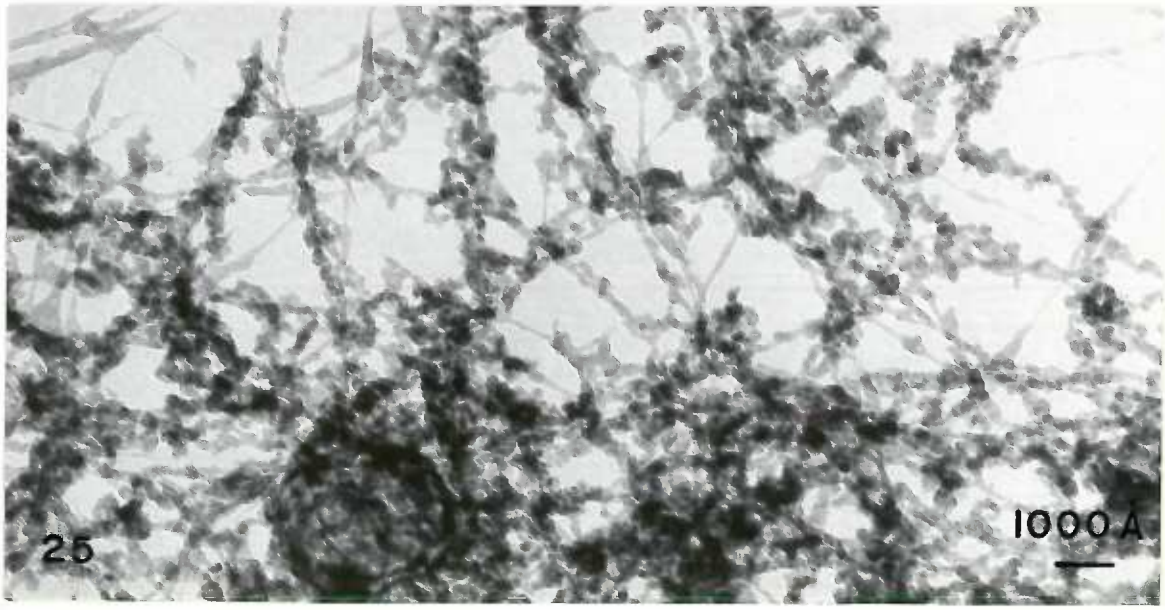

22
5 μ


23
1000 Å

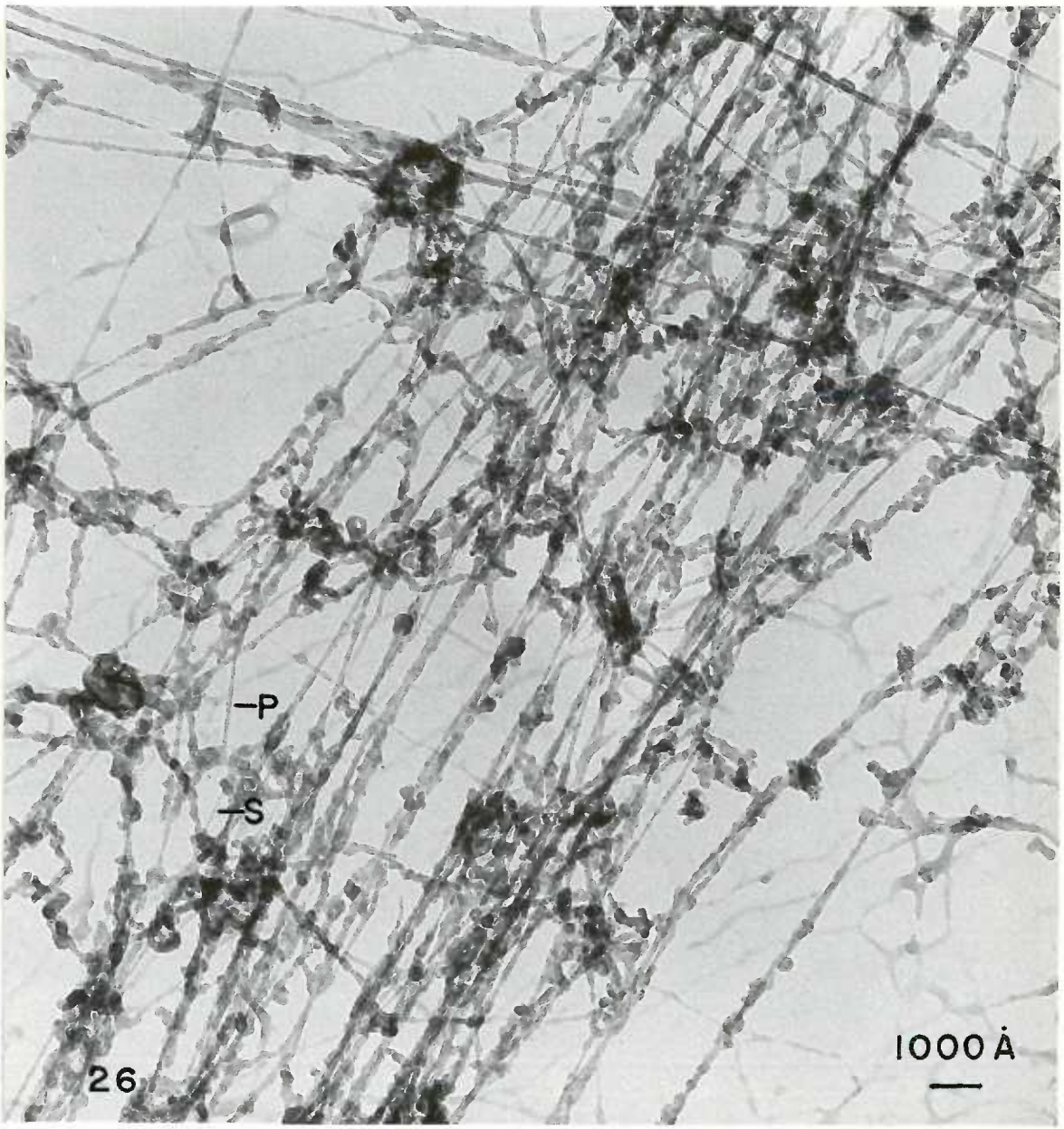
(Figure 24). This dissociated interphase cell was exposed to RNAase. No change in the longitudinal continuity of the chromosome fibers is seen (x6,175).

(Figure 25). At a higher magnification (Figure 24) the individual chromosome fibers exhibit no morphologic change after RNAase treatment (x73,000).


24
5 μ

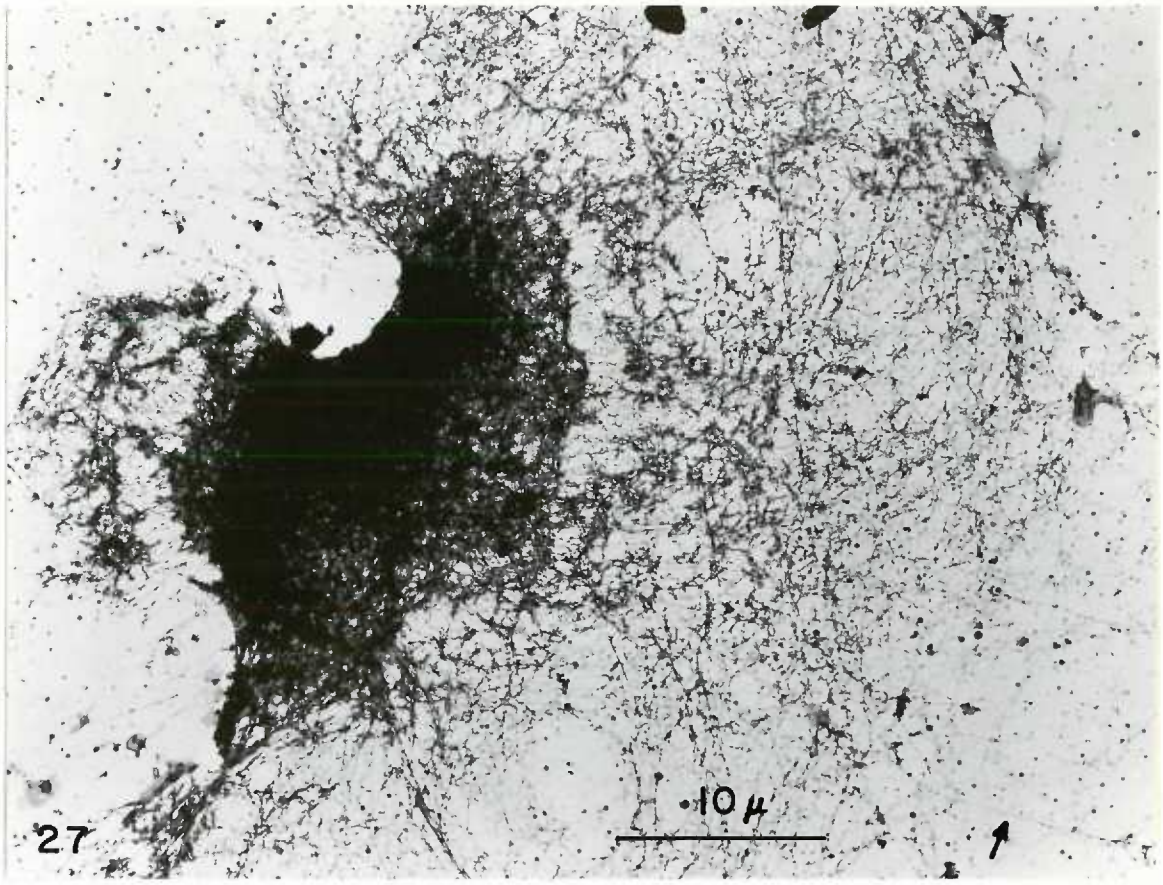

25
1000 Å

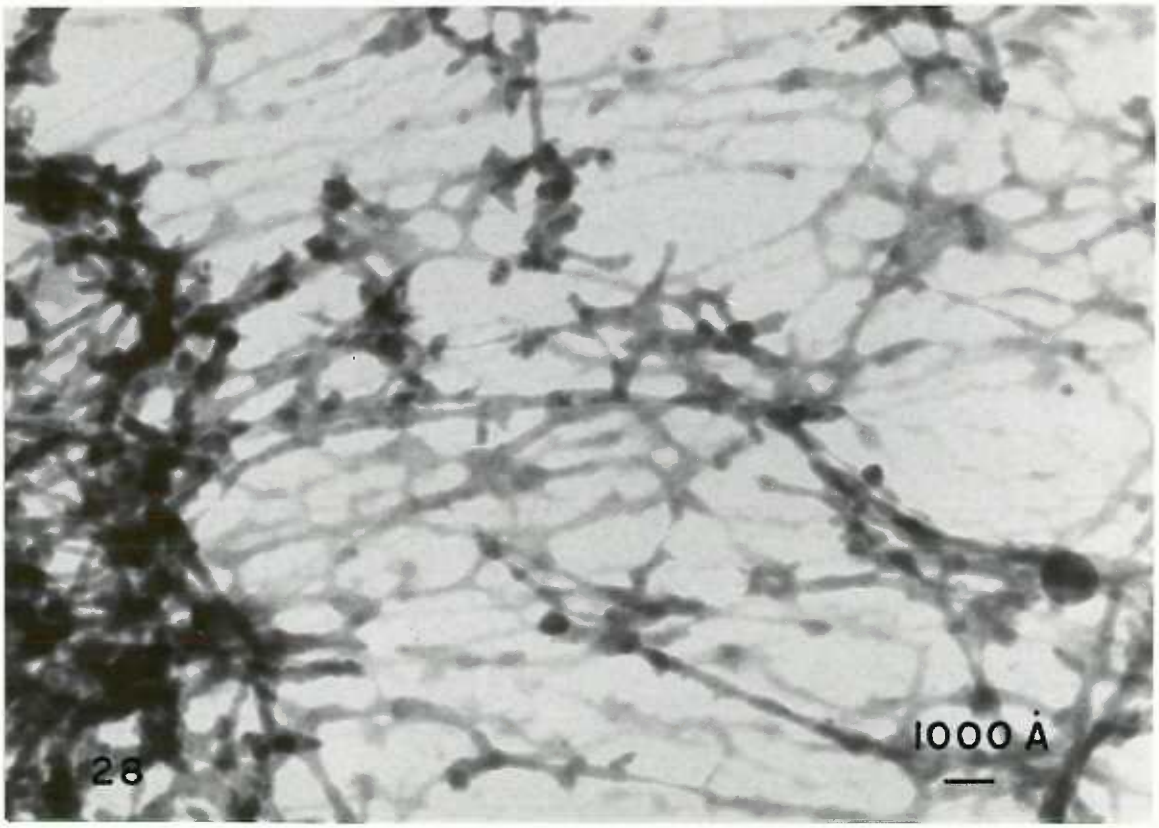
(Figure 26). These chromosome fibers which are from a different cell than Figure 24 were also exposed to RNAase. No change in fiber morphology is evident; 66 Å (S) and 112 Å fibers are seen (x73,000).


—P
—S
26
1000 Å

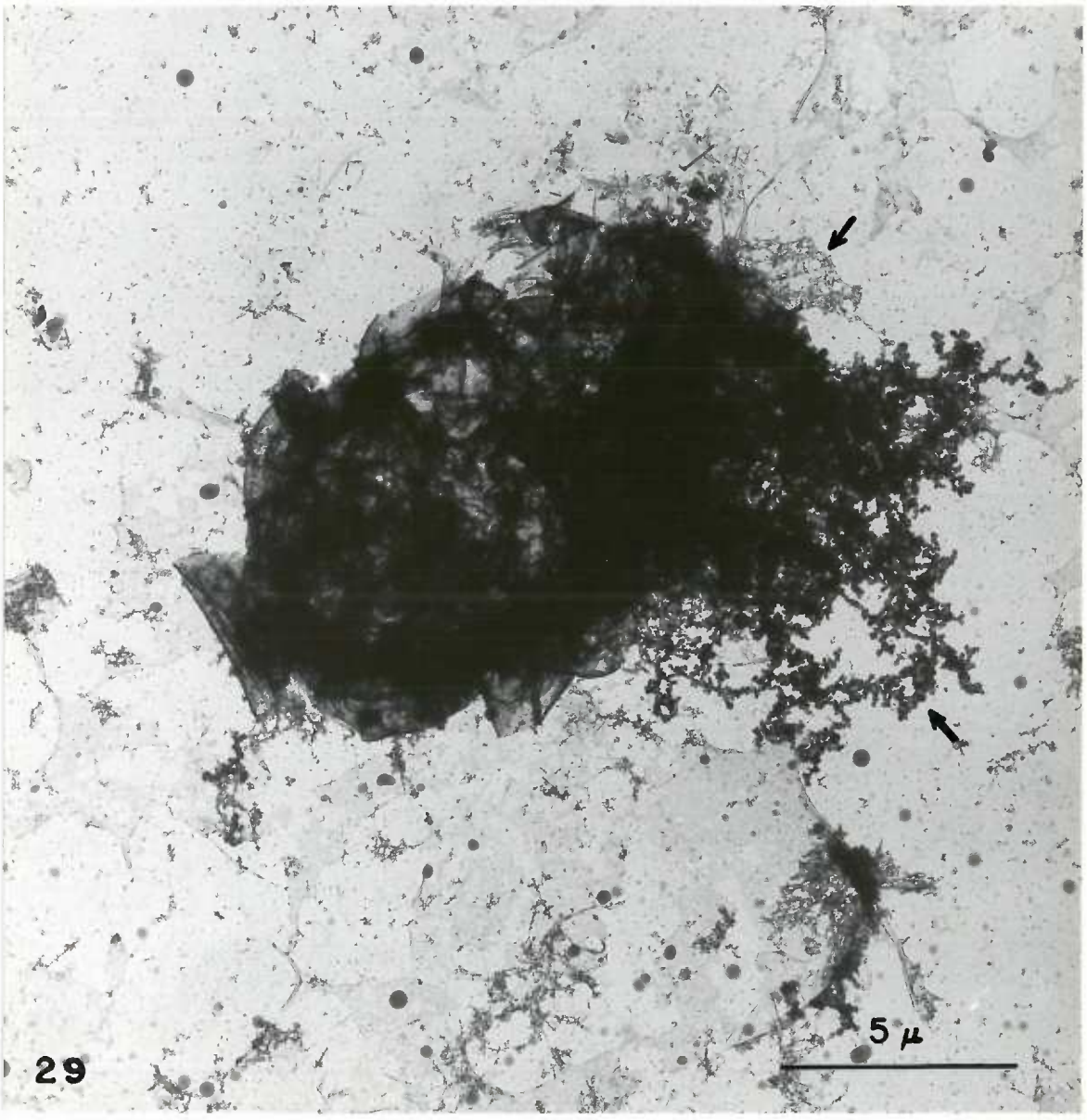
(Figure 27). The interphase chromosomes of this cell were treated with trypsin. There is no disruption of the longitudinal continuity of the fibers (arrow) (x2,670).

(Figure 28). At a higher magnification (Figure 27) it is seen that the chromosome fibers have very little coiling after exposure to trypsin. There is no segmental or overall decrease in the fiber diameter (x60,219).

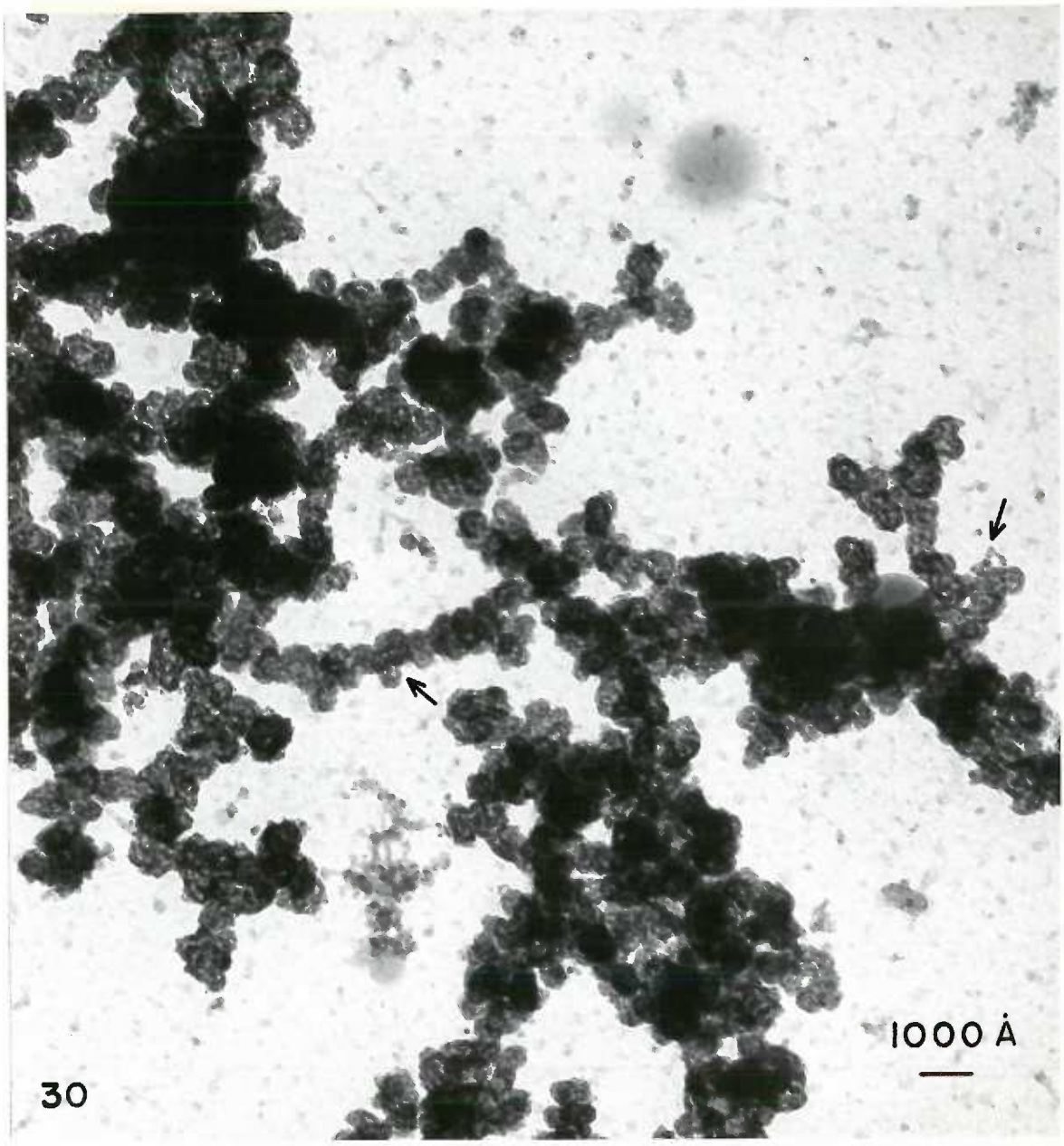

10 μ
27


1000 Å
28

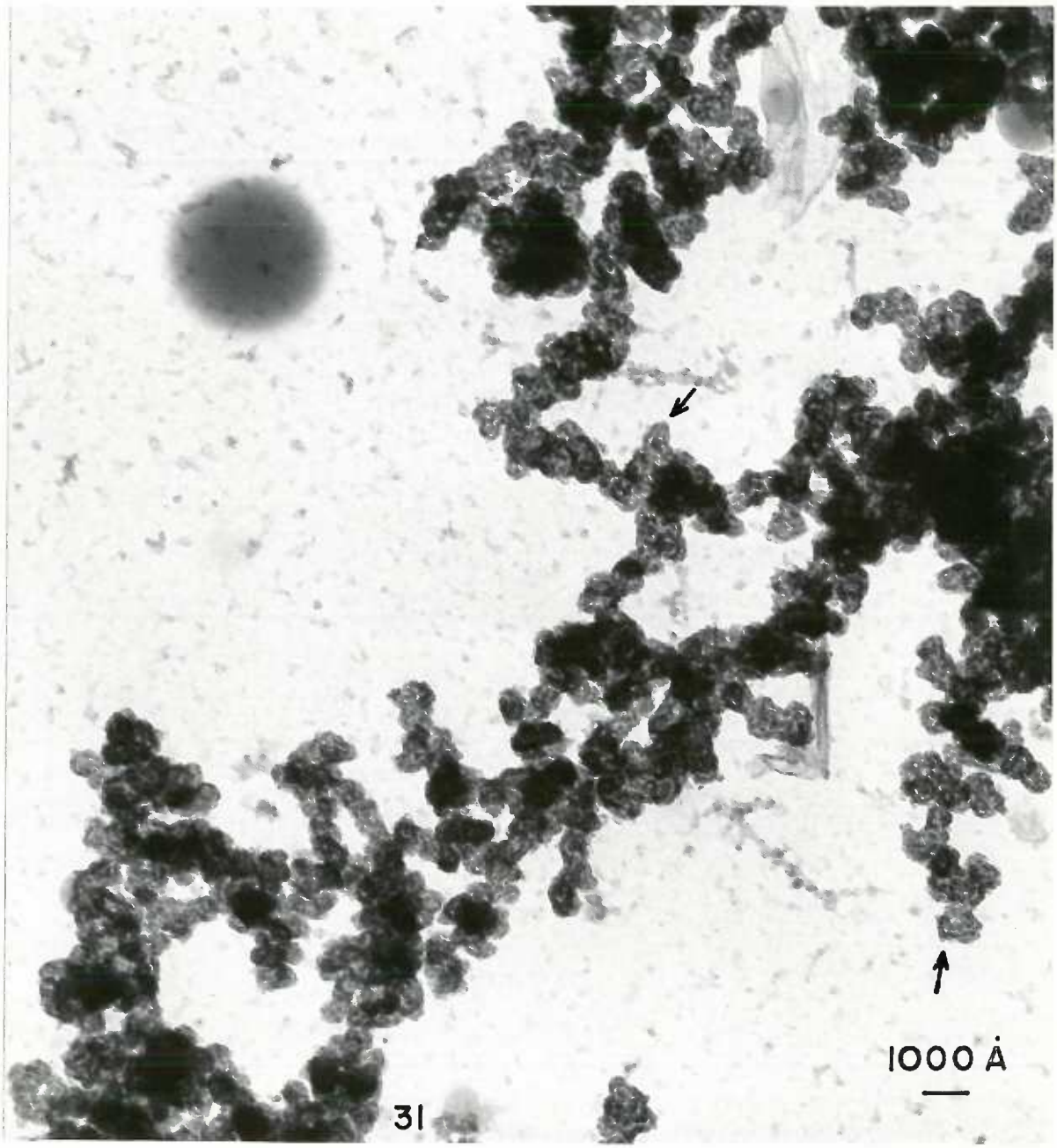
(Figure 29). This cell was suspended in trypsin (rather than KCl) for dissociation. Chromosome fibers which exhibit smaller (upper right) and larger (lower right, right middle) degrees of packaging and condensation are present (x6,650).


29
5 μ

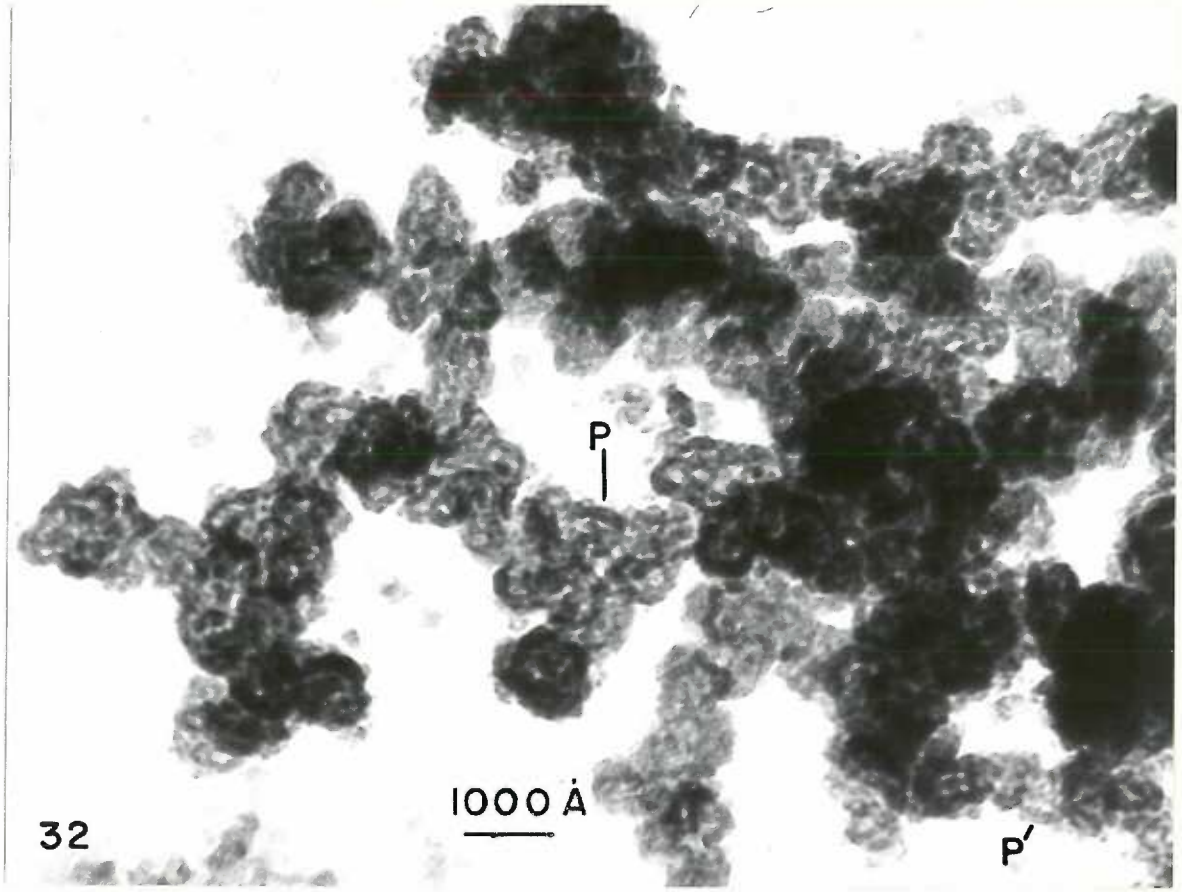
(Figure 30). In some areas (Figure 29) chromosome fiber associations are loosened sufficiently to discern patterns of fiber organization. The configurations suggest that a long continuous fiber of about 112 Å diameter (arrows) is coiled, folded and looped and that such an aggregate is again coiled (secondary coiling) and looped (x70,700).

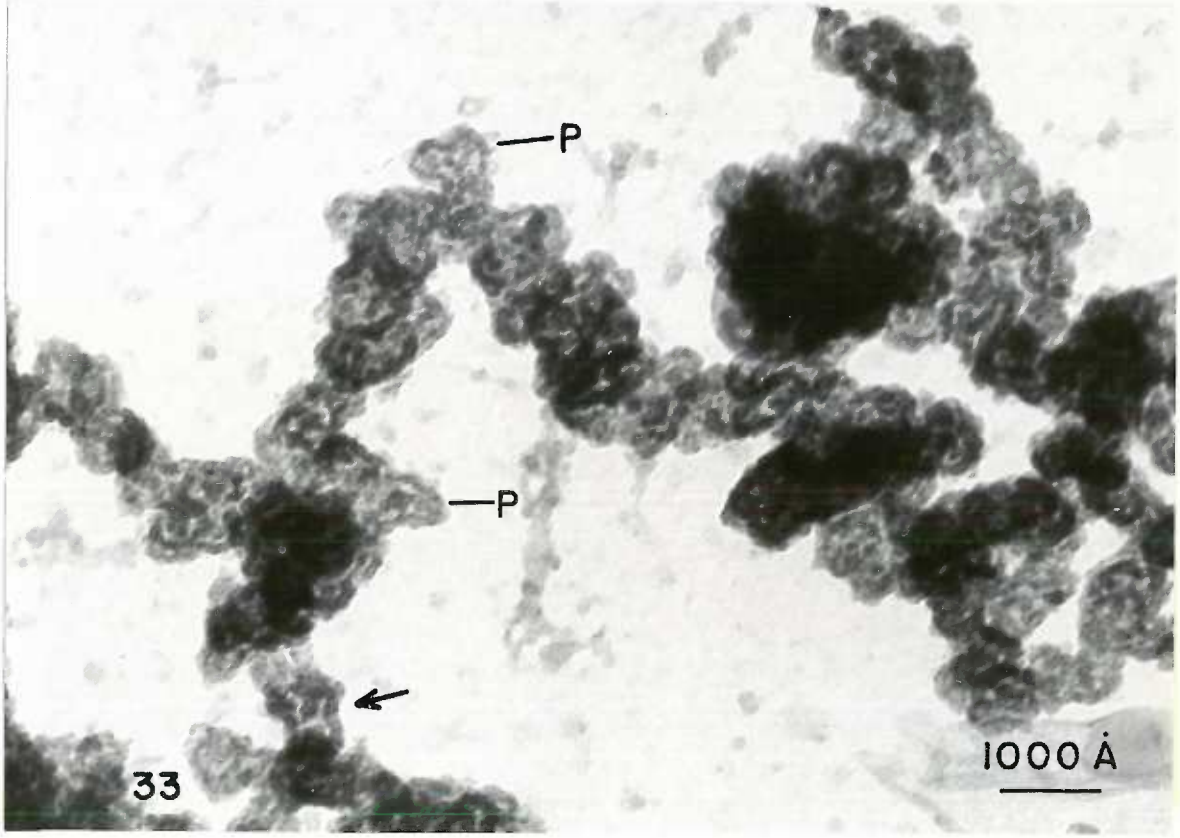

1000 Å
30

(Figure 31). In some areas (Figure 29) chromosome fiber associations are loosened sufficiently to discern patterns of fiber organization. The configurations suggest that a long continuous fiber of about 112 Å diameter (arrows) is coiled, folded and looped and that such an aggregate is again coiled (secondary coiling) and looped (x70,700).


1000 Å
31

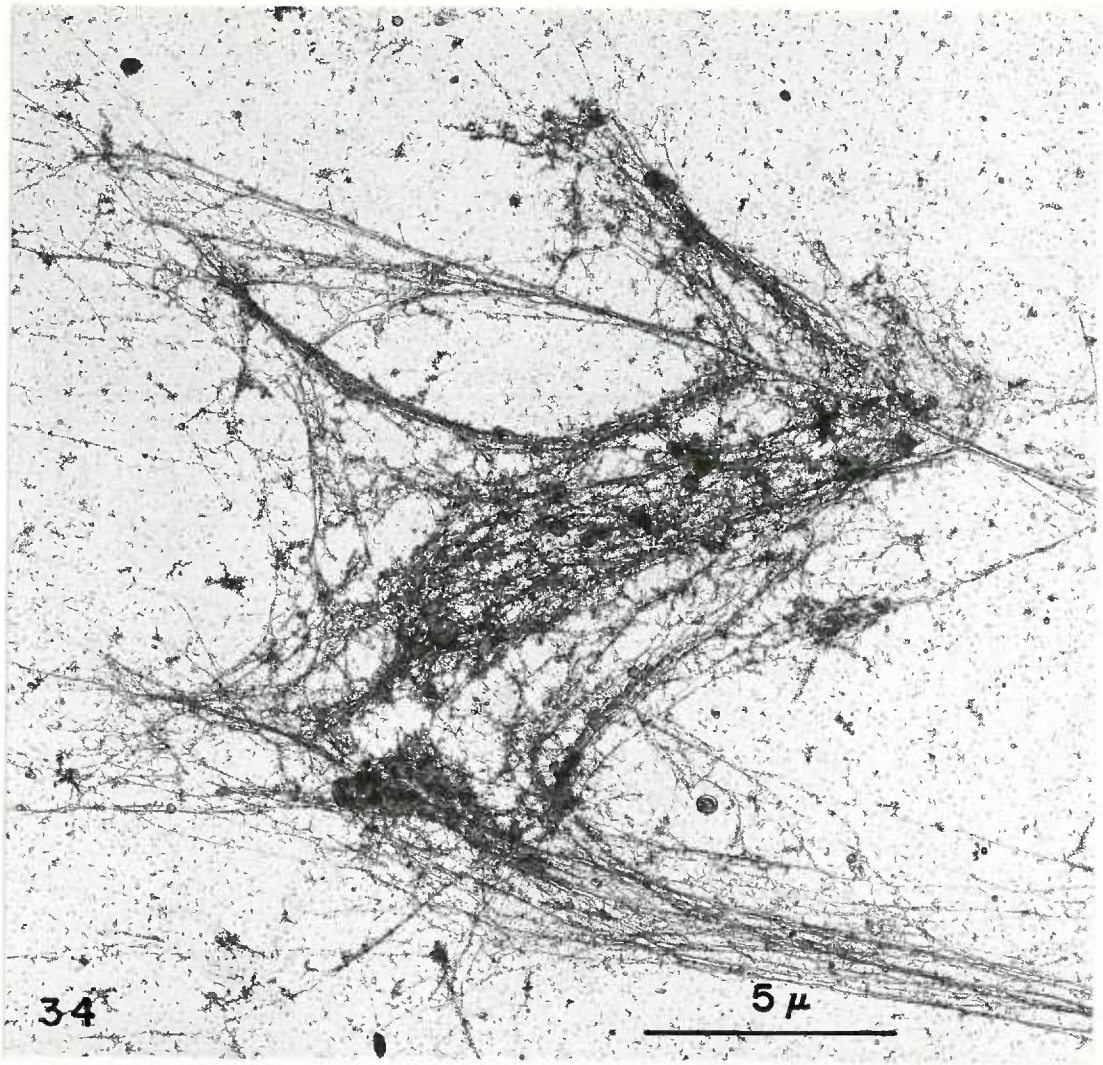
(Figures 32 & 33). The 112 Å fiber (Figures 30 & 31) is clearly revealed in these micrographs (P). It is highly coiled and folded in most areas, however at one location (Figure 33, arrow) three relatively uncoiled fibers are arranged in parallel (x132,000).

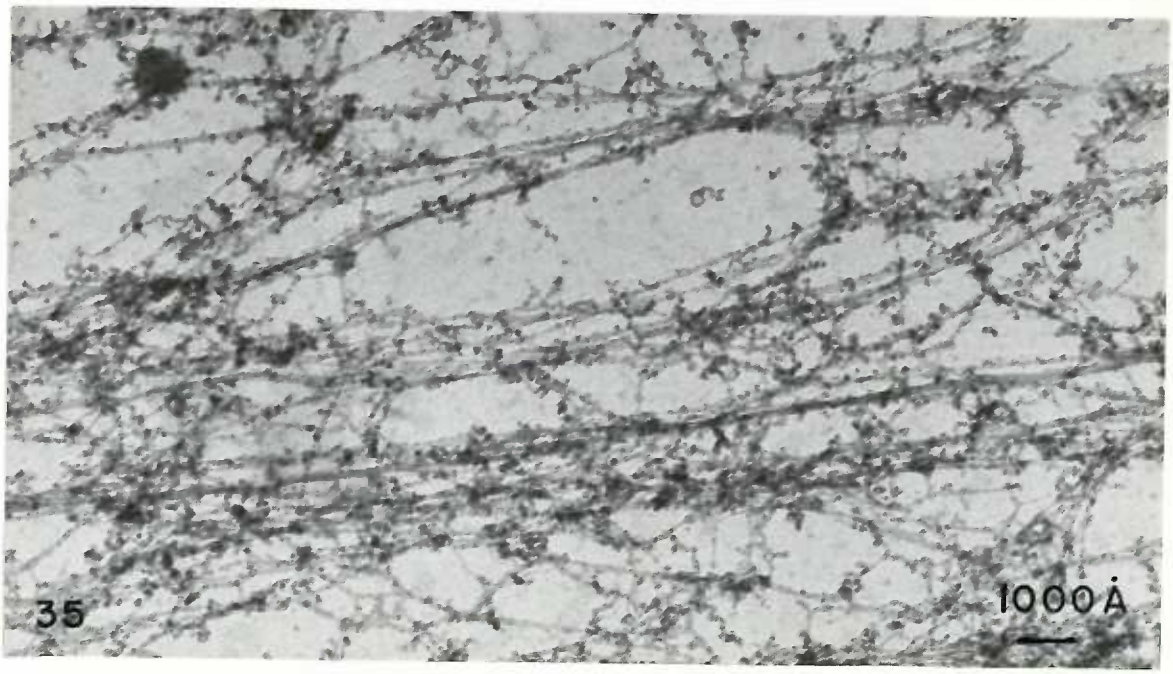

P
1000 Å
32
P'


P
P
33
1000 Å

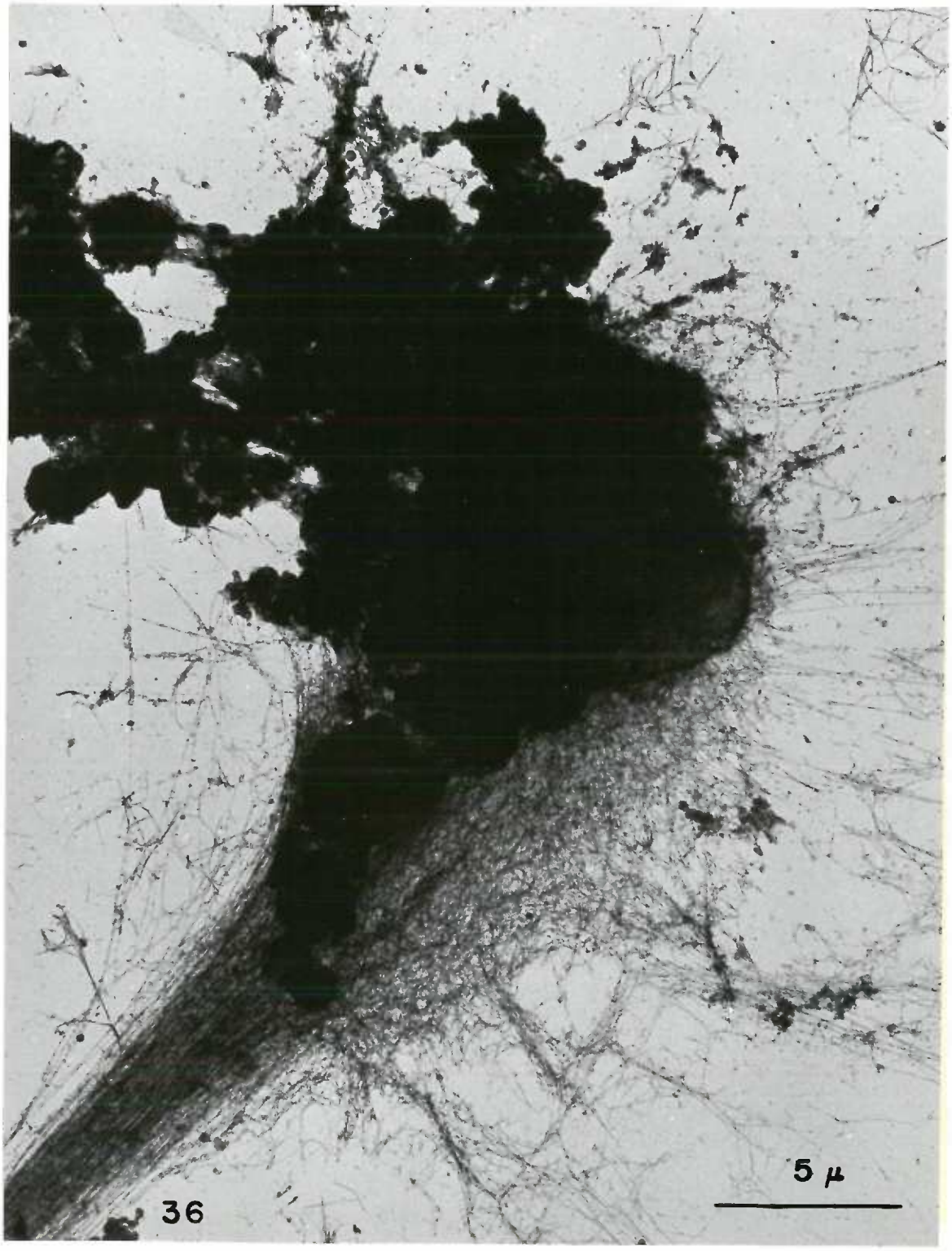
(Figure 34). Following dissociation and spreading these chromosome fibers were treated with chymotrypsin. Numerous fibers are 5 μ long, and no disruption of longitudinal continuity is apparent (x6,650).

(Figure 35). At a higher magnification the chromosome fibers (Figure 34) exhibit a previously unseen form of coiling which give the fibers a beaded appearance. There are suggestions in some fibers of alternating segments of coiling and uncoiling (x72,800).


34
5 μ

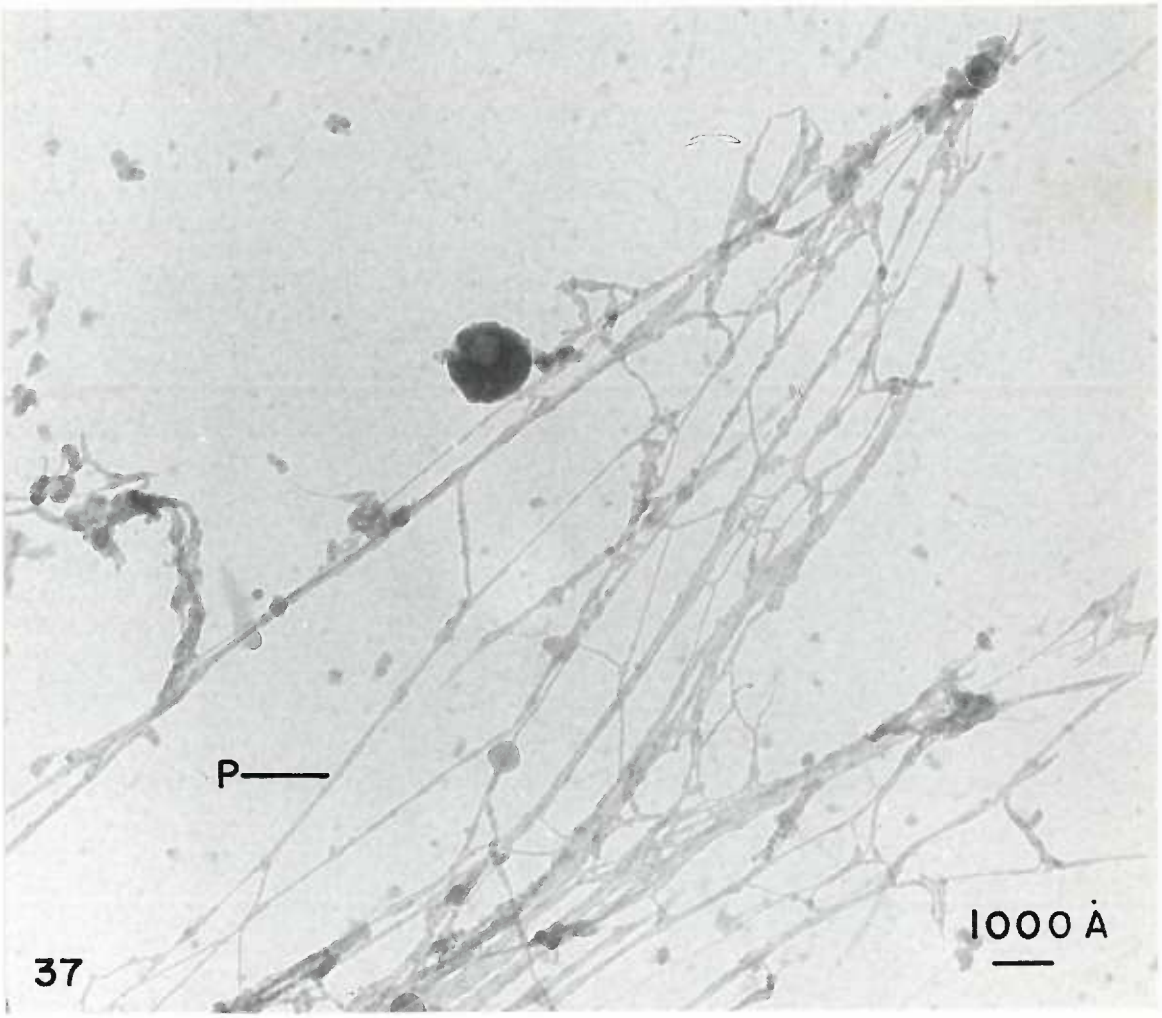

35
1000 Å

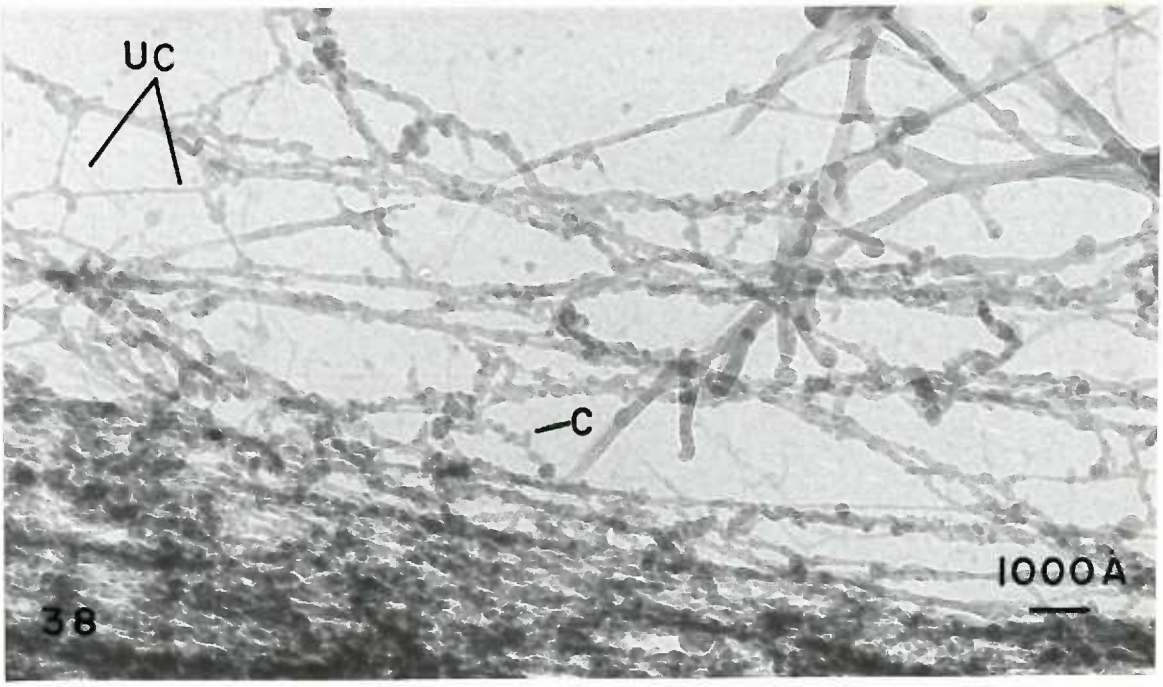
(Figure 36). This aggregate of dissociated cells was exposed to 2.0M NaCl for 20 minutes. Fibers which extend 5 to 10 μ are seen among the many fibers coming from this clump of cells (x6,020).


5 μ
36

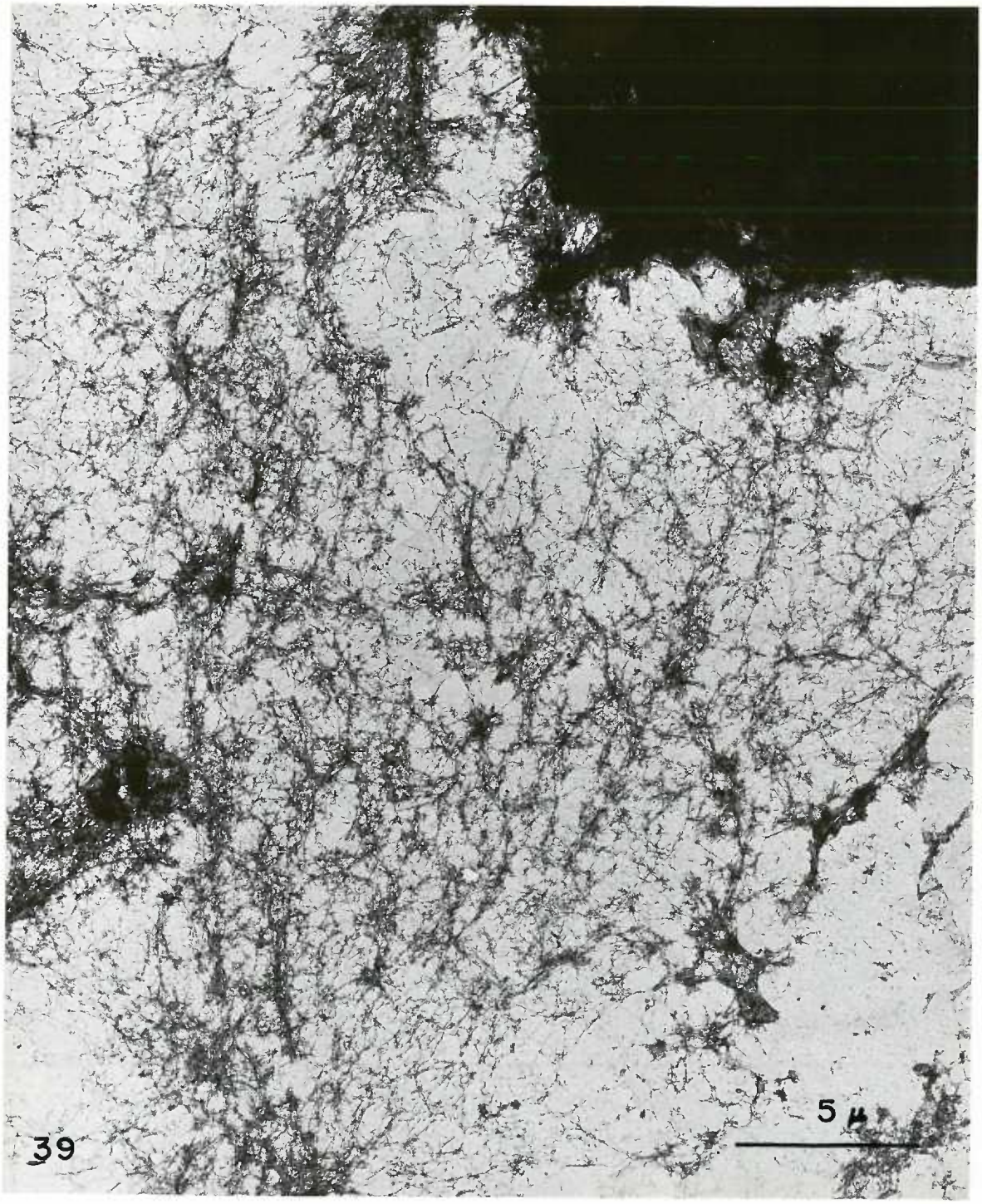
(Figure 37). One portion of Figure 36 contains fibers with almost no coiling. Paired (P) 66 Å fibers are clearly seen (x70,200).

(Figure 38). Another portion of Figure 36 reveals fibers with varying degrees of coiling. Some fibers remain coiled (C) and others are uncoiled (UC) (x70,200).

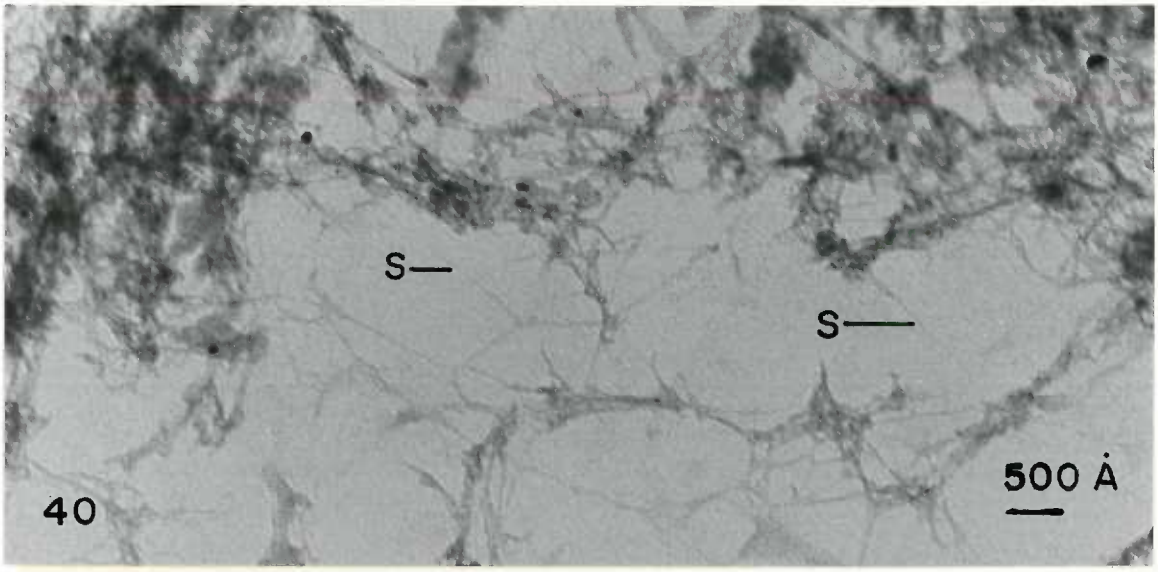

P
1000 Å
37

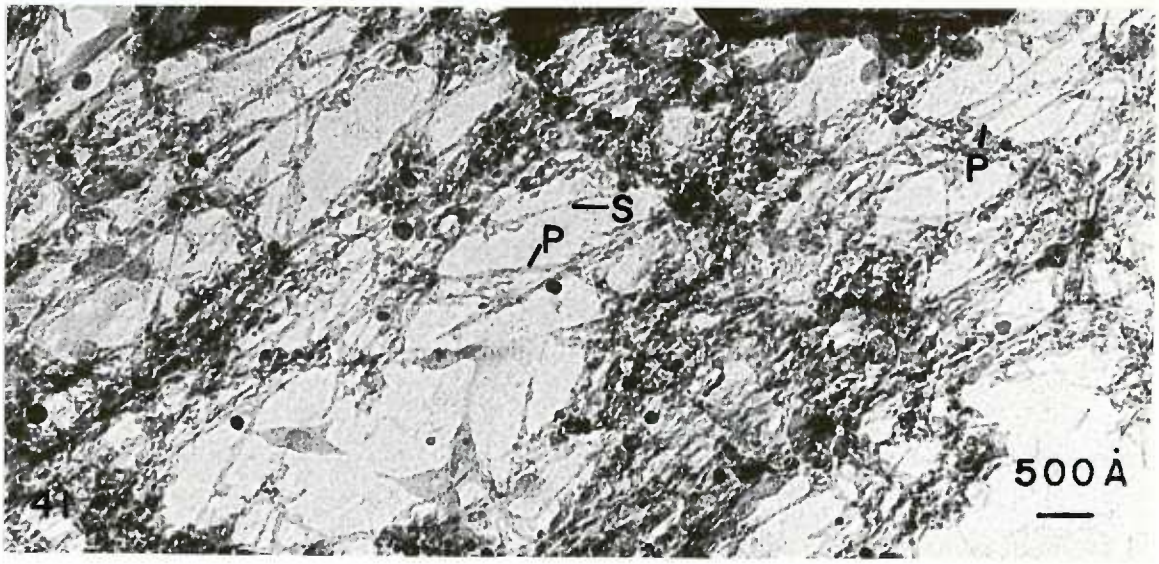

UC
C
1000 Å
38

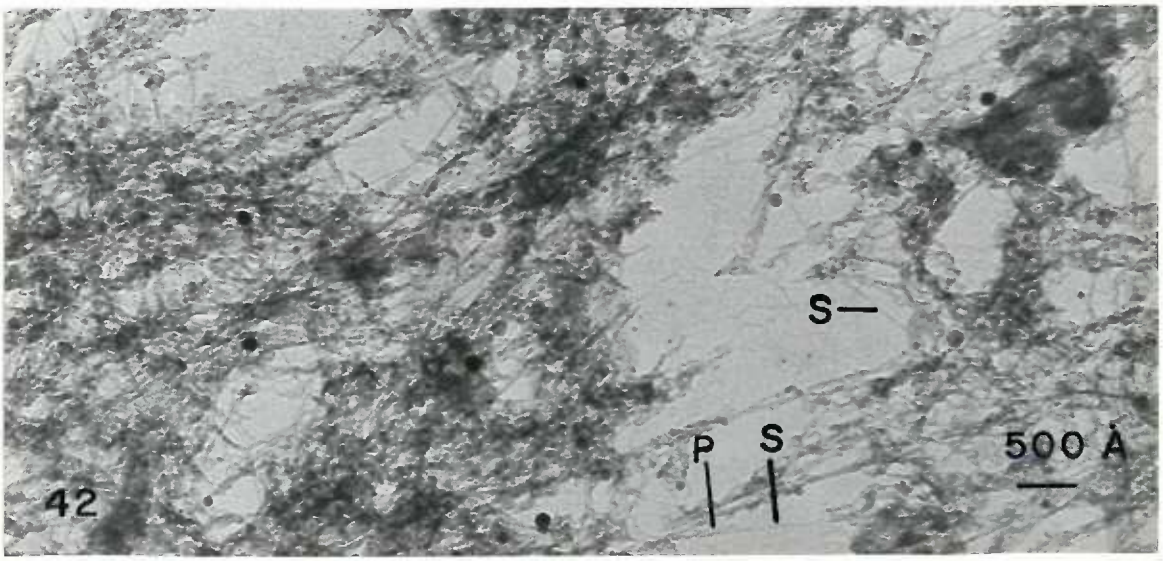
(Figure 39). After dissociation in KCl this interphase cell was treated with 2.0M NaCl for 60 minutes. The chromosome fibers are spread diffusely over a large area, and within this area there are regional concentrations of chromosome fibers (x5,600).


39
5 μ

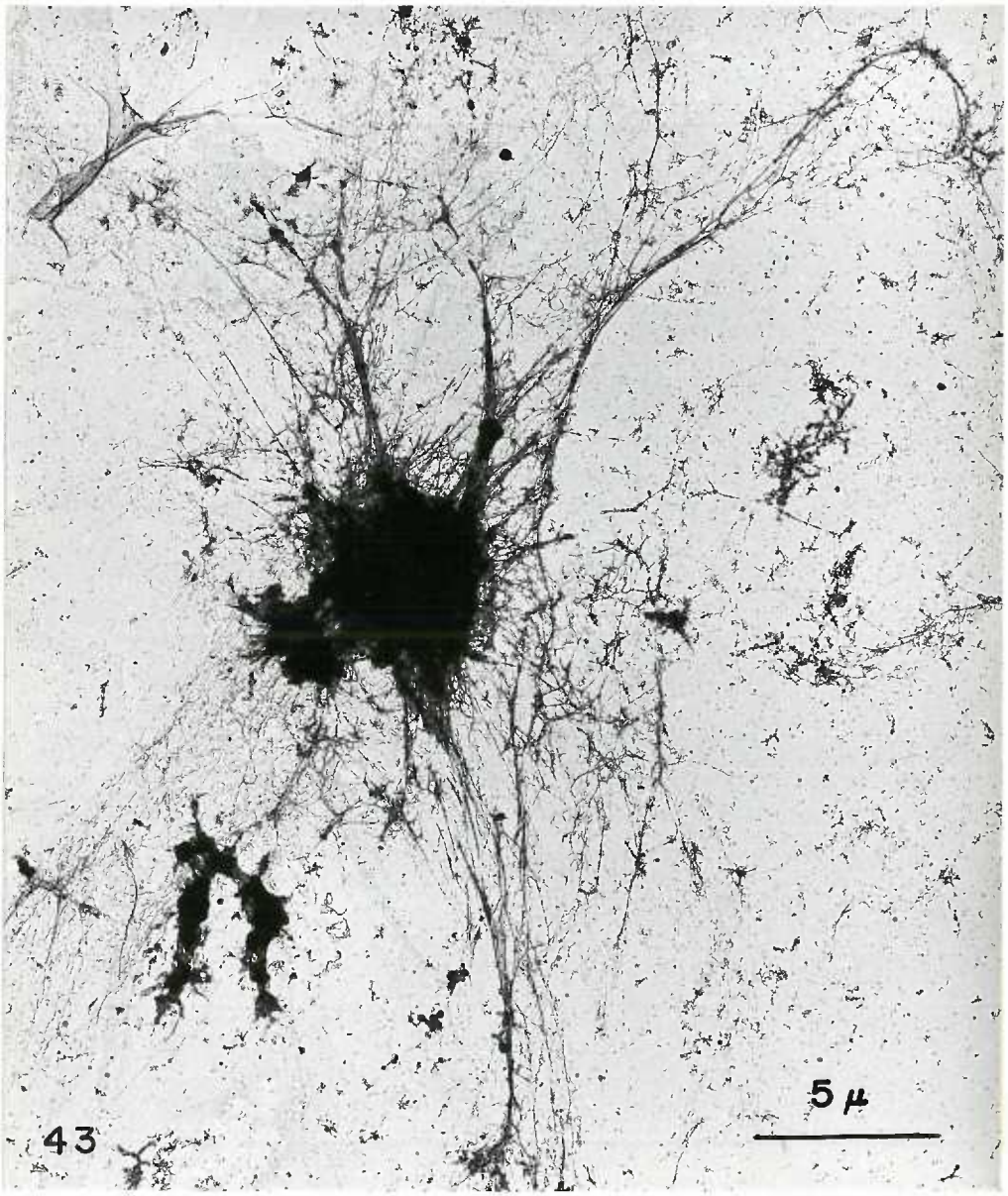
(Figures 40,41,42). These three micrographs are at the same magnification, and they illustrate the range of the 37±6 Å fiber population. Figure 40 contains single (S) 20 to 30 Å fibers. Figure 41 has both paired (P) and single (S) 37 Å fibers. Figure 42 also illustrates paired (P) and single (S) 37 Å fibers (x136,140).


S
S
500 Å
40

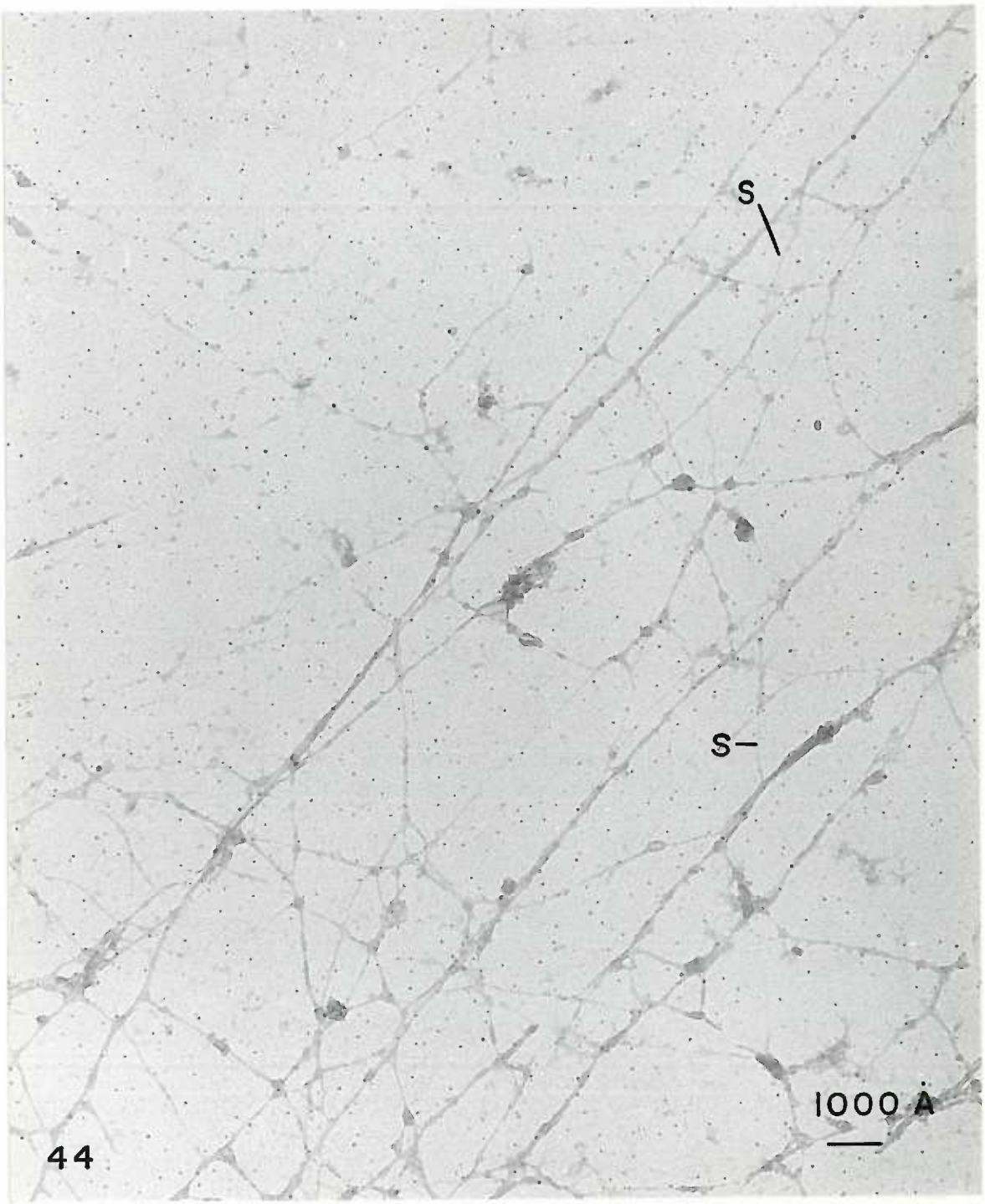

P
S
P
500 Å
41


S
P
S
500 Å
42

(Figure 43). After dissociation this interphase lymphocyte was exposed to 0.2N HCl. The longitudinal continuity of the fibers is preserved (x4,900).

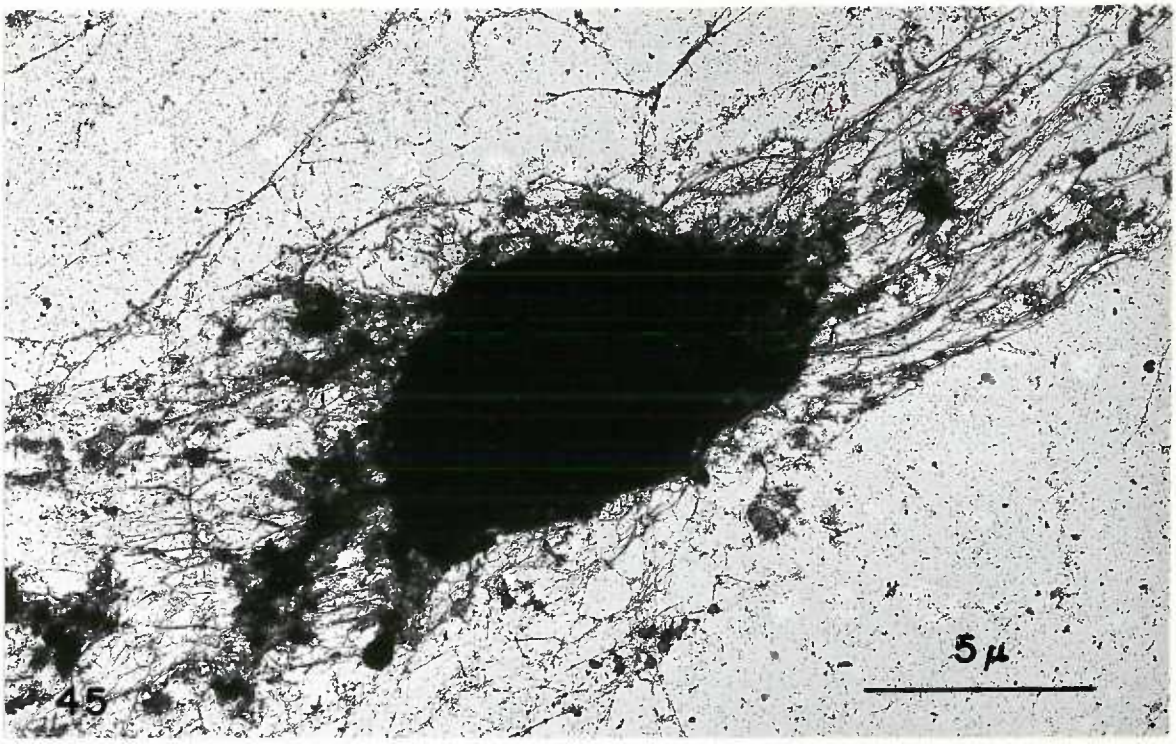

43
5 μ

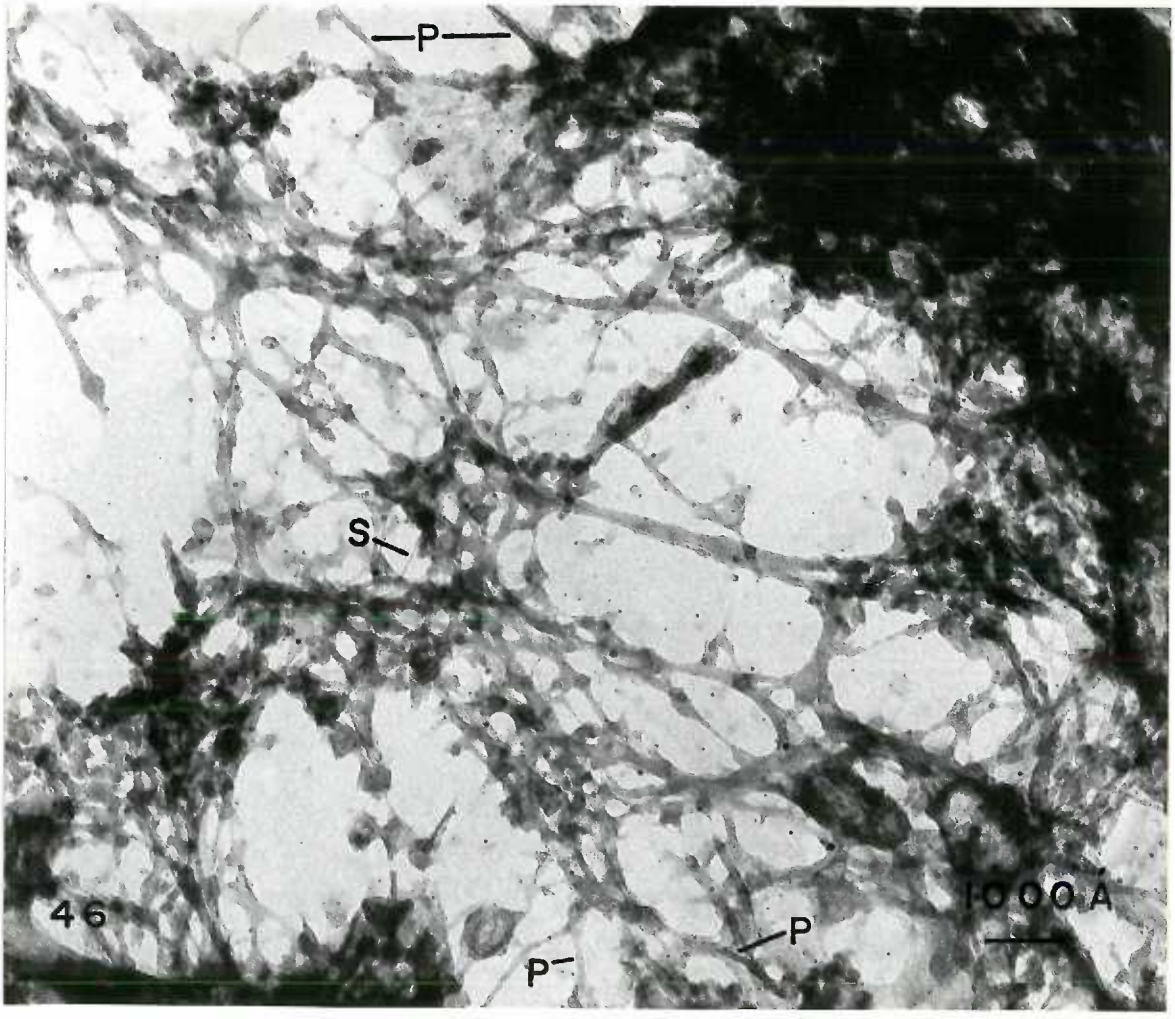
(Figure 44). After dissociation in KCl these chromosome fibers were treated with 0.2N HCl. They exhibit almost no coiling, and their longitudinal continuity is intact. Fibers of the 60 Å (S) population are readily apparent (x84,000).


S
S
1000 Å
44

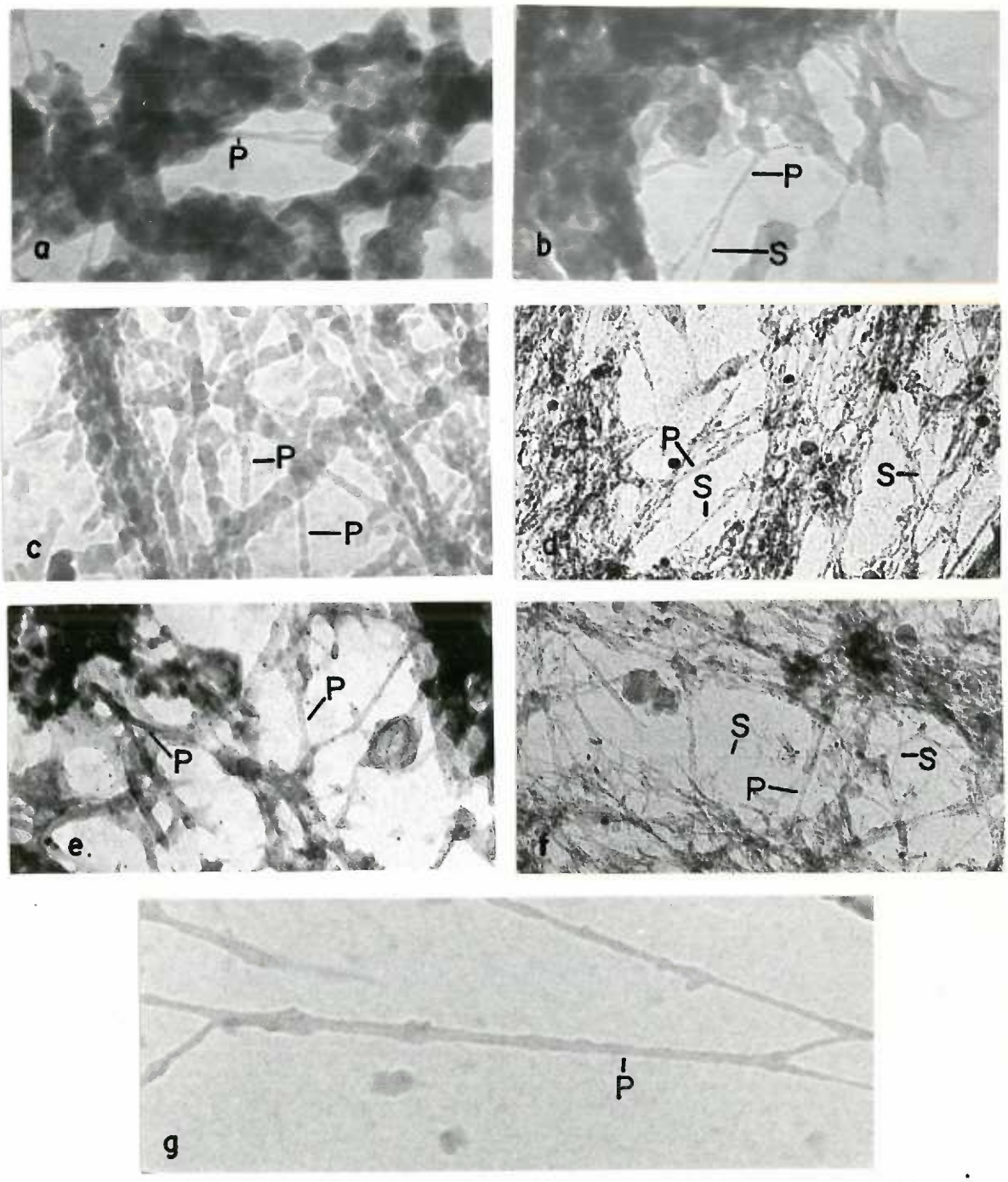
(Figure 45). After dissociation and spreading in KCl this interphase lymphocyte was treated with 0.1M citric acid-0.125M NaCl for 60 minutes and then with 0.2N HCl for 60 minutes. The chromosome fibers are not disrupted (x6,175).

(Figure 46). Higher magnification of Figure 45 reveals that the chromosome fibers have almost no coiling. Fibers that fall within the 37 Å population are present both singly (S) and in pairs (P) (x99,750).


5 μ
45


P
S
P
P
1000 Å
46

(Figure 47). This is a composite which demonstrates close association of fibers in pairs. a, b, and c which are from cells that were dissociated and spread in KCl demonstrate paired (P) and single (S) 66 Å fibers. d and f which are from a cell that was exposed to 2.0M NaCl for 60 minutes subsequent to KCl demonstrate paired (P) and single (S) 37 Å fibers. e which is from a cell that was exposed to 0.1M citric acid-0.125M NaCl followed by 0.2N HCl demonstrates paired (P) 37 Å fibers. g which is from a cell that was exposed to 2.0M NaCl for 20 minutes subsequent to KCl demonstrates paired (P) and single (S) 66 Å fibers (x141,000).



47

500 Å

APPENDIX

SYMBOLS

Treatment

K	= KCl
EDTA	= ethylenediaminetetraacetic acid
UA	= uranyl acetate
PTA	= phosphotungstic acid
ACP	= Anderson critical point drying
DNAase	= deoxyribonuclease
RNAase	= ribonuclease
tryp	= trypsin
chym	= chymotrypsin
glu	= glutaraldehyde
NaCl	= sodium chloride
HCl	= hydrochloric acid
$CaCl_2$	= calcium chloride
cit-Na	= citric acid-NaCl
K_{45}, UA_{10}, etc.	= subscript is length of treatment in minutes
N	= number of cells examined in each group
air	= air drying

Cell

int	= interphase
proph	= prophase
met	= metaphase

Measurement

1, 11, etc.	= cell number; measured with Gaertner microscope
+	= fibers measured but not with Gaertner microscope

Details of the methods are defined in MATERIALS AND METHODS.

APPENDIX

Experimental Treatment	Donor	Cell: culture time(hr)/arrest time(hr)/mitotic phase	Date	Plate number	Measurement	Figure number
K_{150} to UA_1 to air	M166	48/24/int,proph	5-4-65	37-41 92-99	1[1]	-
K_{120} to UA_5 to air	M166	48/24/int	5-4-65	42-50	-	-
K_{15} to UA_{10} to air	M166	48/24/int	5-15-65	69-73	-	-
K_{30} to UA_{10} to air	M166	48/24/int	5-15-65	74-76	-	-
K_{45} to glu_5 to UA_5 to ACP N = 12	M166	48/24/int	5-20-65	66-68	-	-
K_{45} to UA_{10} to ACP	RCS	59/14½/int,met	6-28-65	100-105	I, IIA, IIIA,B,C	4,5,11,12
$K_{160-180}$ to UA_{10} to ACP	JHB	59/12/int	9-6-65	175-183, 238	VI	1-3
K_{35} to UA_{10} to ACP	RCS	72/19/met	6-1-65	77-85	IA,B	6,7
K_{45} to UA_{15} to ACP	RCS	58/14/int	12-27-65	223	-	-
K_{45} to UA_{15} to ACP	RCS	53½/11½/int	12-29-65	221	-	-
K_{30} to PTA_5 to ACP	RGM	60/13/int	8-5-65	245	-	-
K_{45} to PTA_{25} to ACP	RCS	59/14½/met	6-28-65	106,107, 126-130	IVA	47
K_{180} to PTA_{10} to ACP N = 18	RCS	45/0/int	12-22-65	184	-	-
$NaEDTA_{65}$ to UA_{15} to ACP	RGM	60/13/int	8-5-65	240-243	+	17-21
$KEDTA_{65}$ to UA_{15} to ACP N = 6	RGM	60/13/int	8-5-65	244	-	-
$tryp_{75}$ to UA_5 to ACP N = 4	RGM	60/13/int-proph	8-5-65	246-252	+	29-33
$tryp_{80}$ to $chym_{15}$ to UA_{10} to ACP N = 1	RCS	48/14/int	8-6-65	239[11]	-	-

Experimental Treatment	Donor	Cell: culture time(hr)/arrest time(hr)/mitotic phase	Date	Plate number	Measure-ment	Figure number
K_{30} to $DNAase_{15}$ to UA_3 to ACP	RCS	72/19/int,met	6-1-65	86-91	-	-
K_{45} to $DNAase_{20}$ to UA_{15} to ACP	RCS	58½/14/int	12-27-65	207-211	+	22,23
$K_{60,180}$ to $DNAase_{15}$ to UA_{10} to ACP N = 16	JHB	59/12/int	9-6-65	173,174, 237,238	+	-
K_{45} to $RNAase_{15}$ to UA_5 to ACP	RCS	59/20/int	6-28-65	253	-	-
K_{45} to $RNAase_{60}$ to UA_{10} to ACP N = 4	RCS	58½/14/int	12-27-65	212,213, 216	+	24-26
K_{60} to $tryp_{25}$ to UA_{10} to ACP	JHB	59/12/int,met	9-6-65	157-164	+	27,28
K_{60} to $tryp_{25}$ to PTA_{15} to ACP	JHB	59/12/int	9-6-65	155-156	-	-
K_{60} to $tryp_{15}$ to UA_{10} to ACP N = 9	RCS	29/14½/int	6-28-65	152-153	+	-
K_{60} to $tryp_{15}$ to $chym_{15}$ to UA_{10} to ACP	RCS	48/14/int	8-6-65	239,240	-	-
K_{65} to $chym_{30}$ to UA_{10} to ACP N = 4	RCS	48/14/int	8-6-65	214,215	+	34,35
K_{45} to 1M $NaCl_{15}$ to UA_{10} to ACP	RCS	59/14½/int,met	6-28-65	148-151, 108-125	II	-
K_{45} to 1M $NaCl_{15}$ to PTA_{25} to ACP	RCS	59/14½/int,met	6-28-65	131-147	+	-
K_{70} to 2M $NaCl_{20}$ to UA_{10} to ACP N = 26	JHB	59/12/int,met	9-6-65	165-172	III,IV,V, VA	36-38
K_{180} to 2M $NaCl_{60}$ to UA_{10} to ACP	RCS	45/0/int	12-22-65	193,194 198-206	VII +	39-42 8-10
K_{45} to 2M $NaCl_{150}$ to UA_{10} to ACP	RCS	53½/11½/int,met	12-29-65	195-197	+	-
K_{180} to 2M $NaCl_{60}$ to PTA_{10} to ACP N = 10	RCS	45/0/int	12-22-65	224	-	-

Experimental Treatment	Donor	Cell: culture time(hr)/arrest time(hr)/mitotic phase	Date	Plate number	Measure-ment	Figure number
K_{30} to HCl_{60} to UA_{10} to ACP	RCS	$53\frac{1}{2}/11\frac{1}{2}$/int	12-29-65	217-219	+	44
K_{30} to HCl_{60} to PTA_{10} to ACP	RCS	$53\frac{1}{2}/11\frac{1}{2}$/int	12-29-65	220	-	-
K_{180} to HCl_{60} to UA_{10} to ACP N = 4	RCS	45/0/int,met	12-22-65	233-236	-	43
K_{180} to citric $acid_{60}$ to UA_{10} to ACP	RCS	45/0/int	12-22-65	185-192	+	-
K_{180} to cit-Na_{60} to HCl_{60} to UA_{10} to ACP	RCS	45/0/int	12-22-65	225-228	+	45,46
K_{180} to cit-Na_{60} to HCl_{60} to PTA_{10} to ACP	RCS	45/0/int	12-22-65	229-232	-	-
K_{45} to cit-Na_{120} to HCl_{75} to PTA_{15} to ACP N = 4	RCS	$53\frac{1}{2}/11\frac{1}{2}$/int	12-29-65	222	-	-